UNIVERSITÉ DE STRASBOURG

Ecole Doctorale Sciences de la Vie et de la Santé

Thèse présentée pour l'obtention du titre
de
Docteur de l'Université de Strasbourg

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INCIDENCES A COURT ET A LONG TERME D'UNE OBSTRUCTION NASALE BILATERALE CHEZ LE RAT *Ratus norvegicus*

Soutenue le 27 juin 2011

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REMERCIEMENTS

Je remercie les rapporteurs et les examinateurs d'avoir accepté d'apporter un regard critique à ce travail. Je tiens à remercier **Mrs Bernard THIERY** et **Benoist SCHAAL** pour leurs présences également dans le comité de thèse et qui n'ont cessé de me soutenir. Je tiens à remercier **Mr Pascal AMBROSINI**, qui a accepté d'être membre de ce jury malgré une thématique qui semblait a priori relativement éloignée de la sienne. Je tiens également à remercier **Mr Simon THORNTON** pour son aide dans la rédaction des articles scientifiques. IL n'y aura pas assez de mots pour remercier l'Equipe de Physiologie du Comportement de l'Université de Nancy I. Je tiens en premier lieu à remercier **Marie TRABALON** qui a acceptée de m'accueillir dans son laboratoire et n'a ménagé aucun effort pour que cette thèse soit soutenue. Je la remercie vivement pour sa confiance, son énergie, sa disponibilité et pour son aide. Je tiens également à exprimer ma reconnaissance à mon «père spirituel» **Jean-Marc Martrette** pour son expérience et son savoir-faire sans lesquels l'étude des muscles oro-faciaux n'aurait pas été possible.

Merci au personnel technique de l'équipe pour leur aide, leur soutien et leur présence. Merci à toi **Christiane Tankosic** qui m'à enseigné la rigueur scientifique et qui t'es jamais fatigué à me suivre. A toi **Joëlle COUTURIER** mémoire vivante du laboratoire de Physiologie du Comportement, merci pour ton assistance. A mon ami l'animalier **Jean-Charles OLRY** te remercier ici serai diminuer tous les efforts que tu as consenti pour me soutenir.

Enfin merci à ma **famille** et au **gouvernement gabonais** qui ont financé toutes mes années d'études.

SOMMAIRE

LISTES DES FIGURES

LISTE DES TABLEAUX

LISTE DES ABREVIATIONS

ACTH : Hormone adrénocorticotrope

C : Groupe contrôle

CMH : Complexe Majeur d'Histocompatibilité

CO_2 : Dioxyde de carbone

CRH : Corticolibérine

DA : Digastrique antérieur

Dia : Diaphragme

LN : Levator nasolabialis

MHC : Chaînes lourdes de myosine

MS : Masséter superficiel

O_2 : Oxygène

ON : Groupe obstruction nasale

ORL : Oto-rhino-laryngologie

T4 : Thyroxine

TRH : Hormone Thyréotrope

TSH : Hormone Thyréostimuline

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INTRODUCTION GENERALE

Le nez possède plusieurs fonctions (tableau 1) :

- les fonctions **ventilatoire et thermique**, qui permettent le **conditionnement de l'air inspiré** destiné aux échanges respiratoires, en le filtrant, l'humidifiant et le réchauffant,

- la fonction **sensorielle,** olfactive et gustative,

- la fonction **immunitaire**, éliminant les particules aéroportées (poussières, pollens, champignons..),

- il permet également la régulation de la pression dans l'oreille moyenne via la trompe d'Eustache, ainsi que l'évacuation du surplus de liquide lacrymal,

La fonction des voies respiratoires supérieures est interactive, gérant les adaptations nécessaires à des fonctions aussi multiples et variées que les fonctions liées à la respiration : les pleurs, le rire, la toux, le bâillement, le hoquet, plus tard la phonation, et des fonctions digestives telles la mastication, la succion, la déglutition, voire l'audition par la proximité de l'oreille moyenne (abouchement de la trompe d'Eustache dans le haut pharynx).

Chez le nourrisson humain, la **fonction ventilatoire** utilise uniquement la **voie nasale** dont l'obstruction peut menacer la vie. La ventilation par la voie buccale, excepté lors des pleurs, n'est correctement établie que vers l'âge de 3 à 5 mois.

Fonctions	Structures anatomiques mises en jeu
Ventilatoire	
Voie aérienne supérieure principale	Méats nasaux, nasopharynx
Conditionnement de l'air inspiré	Vaisseaux sanguins, mucus
Thermique	
Homéostasie thermique	Vaisseaux sanguins
Sensorielle	
Olfaction	Epithélium olfactif, Organe septal
Détection des phéromones	Organe vomeronasal
Gustation	Epithélium olfactif voie rétronasale
Phonation	Cavités nasales et paranasales
Détection des substances irritantes	Système trigéminal
Protectrice	
Mise en place de réflexes protecteurs	Système trigéminal
Filtration des particules	Epithélium kératinisé et respiratoire
Barrière immunitaire	Mucus, barrière épithéliale
Secondaires	
Aération de l'oreille moyenne	Trompe d'Eustache
Evacuation du liquide lacrymal	Conduits lacrymo-nasal

Table 1 : Résumé des fonctions nasales et principales structures anatomiques mises en jeu.

L'obstruction nasale est au départ un symptôme banal de consultation médicale, mais celui-ci revêt une grande importance compte tenu de son retentissement nasal et sinusien mais aussi sur le reste de la sphère ORL (les oreilles, le larynx, et le pharynx) (figure 1).

En cas d'obstruction nasale, une suppléance buccale doit s'établir afin de maintenir la fonction ventilatoire.

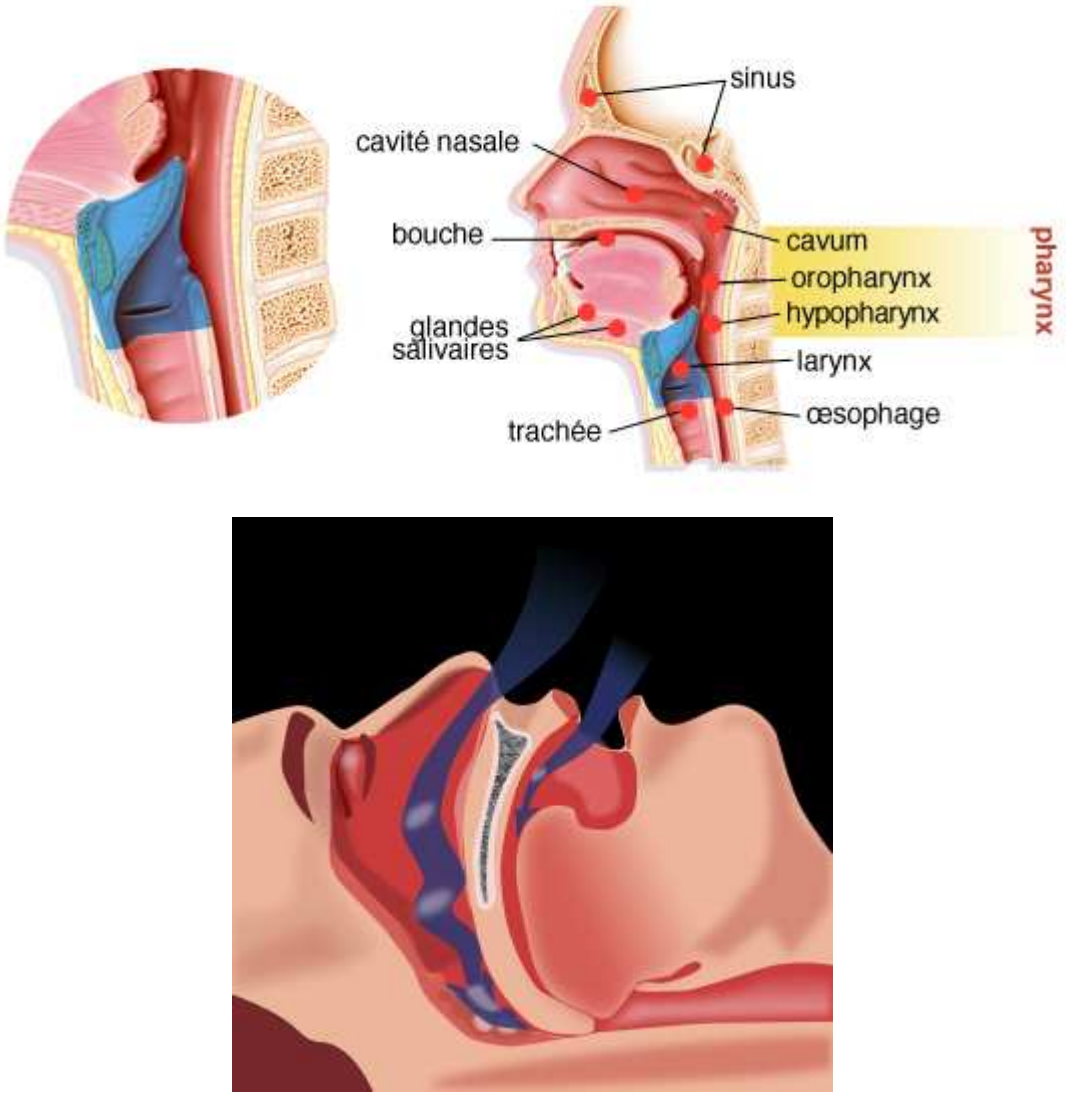

Figure 1 : Voies aéro – digestives supérieures chez l'enfant.

L'obstruction nasale de l'enfant est un problème très fréquent et sous-estimé. Chez le nouveau-né et le nourrisson, seule, la respiration nasale est fonctionnelle. En effet, pendant les 3 premières semaines de vie, le nourrisson ne respire exclusivement que par le nez du fait de l'anatomie pharyngo – laryngée, qui comporte un voile descendant jusqu'au niveau de l'épiglotte. Les voies aériennes et alimentaires sont anatomiquement et fonctionnellement distinctes, ce qui permet au nouveau-né d'effectuer simultanément les fonctions de respiration et d'alimentation sans risque de fausse route. On comprend donc qu'une obstruction nasale bilatérale peut être responsable d'une détresse respiratoire.

Lors d'une obstruction nasale, le flux aérien présente un trajet à concavité inférieure. L'air inspiré pénétrant dans l'orifice narinaire prend une direction oblique en haut et en arrière, traverse la valve nasale constituée par la cloison et le bord inférieur du cartilage triangulaire. Il glisse ensuite sur la tête du cornet inférieur et vient heurter la tête du cornet moyen ; une faible part du courant aérien pénètre la fente olfactive, la majeure partie emprunte le méat moyen jusqu'aux choanes. L'air expiré suit pratiquement la même voie, une partie de l'air emprunte cependant le méat inférieur. On conçoit la gêne respiratoire que peut apporter un obstacle architectural ou muqueux se situant sur le trajet des courants aériens. De plus, le nouveau-né est quasiment incapable de respirer par la bouche parce qu'en raison de la position haute du larynx, l'épiglotte est quasi en continuité avec le palais mou, et la langue est très proche du palais.

En résumé, l'obstruction nasale du nouveau-né engendre donc des troubles respiratoires qui sont majorés pendant la tétée et soulagés par les cris. Cette obstruction nasale s'associe parfois à des troubles de la déglutition avec fausses routes. Elle va entraîner de nombreux troubles variables en importance selon la durée, la sévérité et la suppléance de la respiration buccale. Les répercussions d'une obstruction nasale peuvent être locales, régionales ou générales (figure 2).

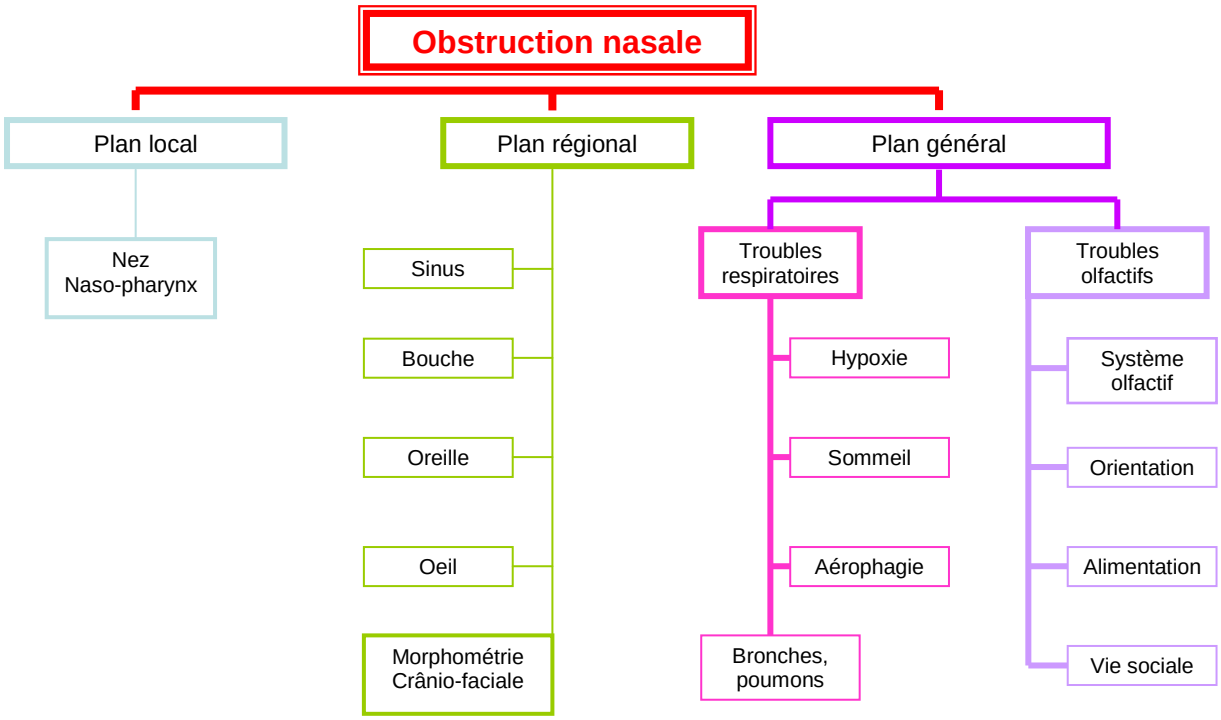

Figure 2 : Structures et comportements susceptibles d'être touchés par l'obstruction nasale.

*** Au niveau local**, l'obstruction nasale entraîne une stase des sécrétions, un mauvais drainage mucociliaire, ce qui occasionne une élimination défectueuse des particules exogènes et des agents infectieux.

*** Au niveau régional**, l'obstruction nasale entraîne une mauvaise aération de l'oreille moyenne *via* la trompe d'Eustache et des sinus. La suppléance par la respiration buccale entraîne une sécheresse buccale, et à long terme des troubles de la croissance du tiers moyen de la face, avec un faciès adénoïdien classique (béance labiale et de l'articulé dentaire, palais étroit et ogival) (figure 3).

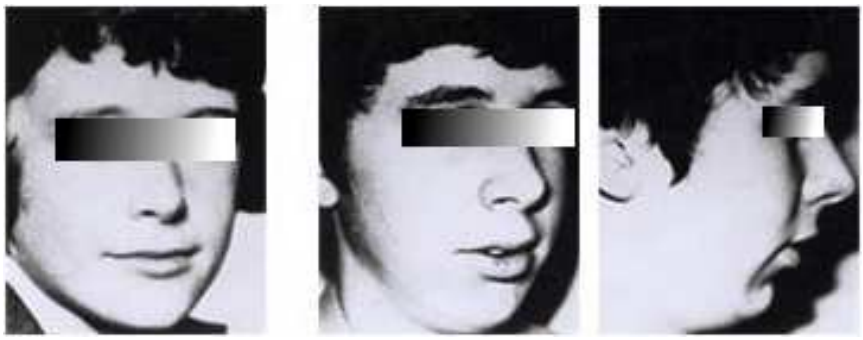

Figure 3 : **A gauche**: un garçon de 10 ans au développement normal, on observe une excellente posture orale et une face équilibrée. **Au centre et à droite**: même garçon âgé de 17ans après la mise en place de la respiration buccale, on observe un allongement de la face et un retrait de la mâchoire inférieure (planète orl 1998)

* Enfin, **sur le plan général**, l'obstruction nasale peut entraîner des **troubles de la croissance** par insuffisance respiratoire chronique, **troubles du comportement** à cause du sommeil perturbé et de la fatigue diurne, **troubles de l'appétit** par **diminution de l'odorat et du goût**. En effet comme tous les autres Mammifères, l'homme est capable de détecter et de différencier très efficacement de multiples molécules odorantes. Ses capacités d'apprentissage des sensations olfactives, lui permettent d'ajouter sans cesse de nouvelles informations dans un contexte de souvenirs et de vie relationnelle.

Chez les Mammifères, dès les premières étapes de l'ontogenèse, la perception et la cognition olfactives sont organisées par plusieurs processus : l'un repose sur des mécanismes héritables de perception d'informations chimiques ; l'autre est fondé sur l'acquisition d'informations qui sont soit invariables au niveau de l'espèce, soit

variables au gré des fluctuations de l'environnement individuel. Les mécanismes chimiosensoriels prédéterminés et les mécanismes physiologiques sont probablement variables en fonction des contraintes auxquelles l'individu doit faire face.

Les compétences fonctionnelles de l'olfaction chez les Mammifères sont adaptées dans un premier temps aux actions vitales du nouveau-né. Leur impact se fait sentir sur trois fonctions essentielles : localiser la mamelle, réduire les pertes énergétiques en reconnaissant sa mère et apprendre les signaux annonçant des états de détresse et d'insécurité. Les actions osmoguidées se manifestent de façon « passive » par des mouvements oraux - faciaux, et de façon active par des mouvements céphaliques ou segmentaires. Ces réponses oro-faciales sont construites dans l'interaction entre un système de détection sensorielle et un environnement chimique (odeurs) donné. La forme de l'espace affectif des odeurs se construit dans de nombreux contextes sociaux ou alimentaires. Elle dépend également de l'expérience olfactive du sujet ainsi que de son état interne momentané.

Dans un deuxième temps**,** la régulation de la structure sociale du groupe est en relation avec le niveau d'émotivité de chaque individu face aux contraintes environnementales. Ce niveau d'émotivité peut être communiqué par voie olfactive, ce qui permet à chaque individu d'identifier chacun de ses congénères, non seulement en tant qu'individu appartenant à la même espèce, mais aussi en tant qu'individu occupant telle ou telle position sociale dans le groupe. La charge émotionnelle que l'individu associe aux odeurs qu'il perçoit, va alors le motiver à interagir ou non, avec l'un de ses congénères. Ainsi Hepper (1987) montre qu'un rat âgé de quelques jours, préfère se rapprocher d'un de ses frères côtoyé depuis la naissance, plutôt que d'un autre frère inconnu, lorsque la portée a été répartie depuis la naissance entre des mères adoptives. Quand le jeune rat doit choisir entre deux congénères inconnus, dont l'un est son frère génétique, alors que l'autre provient d'une autre portée, il choisit son apparenté. Tout se passe comme si le choix se portait sur le congénère qui présente les marqueurs les plus proches de la représentation de référence. Hepper a montré avec d'autres expériences, que le modèle de référence est une combinaison d'odeur correspondant à son marqueur génétique, et de l'apprentissage précoce des odeurs de congénères qu'il a pu acquérir au cours de son développement. Des données neuroéthologiques montrent l'existence d'une image olfactive acquise dans l'épithélium et le bulbe olfactif (Mouly *et al.*, 1990,

1993). Cette image olfactive semble jouer une fonction dans le comportement social de l'individu : des souris bulbectomisées montrent une baisse des activités sociales (Means *et al.,* 1984). Le complexe majeur d'histocompatibilité (CMH) détermine en partie l'identité olfactive des individus. En effet, des molécules solubles du CMH ont été retrouvées dans les fluides corporels (urine, sérum, salive, sueur) de nombreuses espèces (Singh *et al.,* 1988). Ces molécules constituent une source d'odeurs qui assure la reconnaissance entre individus et influence leur comportement social et sexuel. Hurst *et al.,* (2001) ont démontré que l'acuité olfactive des rongeurs, leur permettait de distinguer des urines de souches qui diffèrent uniquement par les gènes du CMH.

En résumé, au cours de la période postnatale, une obstruction nasale bilatérale chez un nourrisson est susceptible d'entraîner :

- une détresse respiratoire en altérant le conditionnement de l'air inspiré.
- des perturbations nutritionnelles et hydriques.
- une privation sociale partielle en perturbant l'orientation vers la mère et les congénères.

Ces différents effets sont connus pour leur capacité à stimuler **la réponse neuro-endocrine au stress**. Dans le cas où elle se traduirait effectivement par la mise en place d'une réponse au stress, l'obstruction nasale bilatérale représenterait une situation stressante chronique, c'est-à-dire une perturbation relativement modérée se prolongeant sur plusieurs jours (Pacak & Palkovits, 2001).

Selye (1950) fut le premier à proposer une véritable théorie concernant **le stress** et ses effets sur l'organisme. Cette théorie, connue sous le nom de Syndrome Général d'Adaptation, comprend trois étapes successives :

* la première étape correspond à une **phase d'alarme ou phase initiale** de la réponse. Elle correspond au choc physiologique suivant la perception du facteur de stress.

* la phase d'alarme est suivie par la **phase de résistance ou phase d'adaptation**, au cours de laquelle l'organisme tente de rétablir son homéostasie.

* si la perturbation est trop importante, l'équilibre ne peut pas être rétabli, et l'organisme entre alors dans une **phase d'épuisement** conduisant à l'apparition de divers effets délétères.

Aujourd'hui, le terme de "réponse au stress" désigne un ensemble de réactions cognitives, comportementales et physiologiques, permettant de maintenir l'homéostasie d'un organisme soumis à une situation défavorable (Lazarus & Folkman, 1984; Chrousos, 1998a). Les réactions physiologiques font intervenir deux systèmes neuroendocriniens couplés mais relativement autonomes: l'axe catécholaminergique, qui intervient principalement dans la phase initiale de la réponse (phase aigüe), et l'axe corticotrope, particulièrement impliqué dans la phase de résistance (phase chronique) (Chrousos, 1998b).

Sous l'effet d'un stimulus de stress, l'hypothalamus entraine la libération des catécholamines au niveau de la médullosurrénale : la noradrénaline et l'adrénaline. La noradrénaline est libérée indépendamment de l'adrénaline. Ces deux hormones vont entraîner :

* une dégradation du glycogène en glucose pour augmenter la glycémie,
* une augmentation de la fréquence cardiaque et de la pression artérielle,
* une augmentation de la fréquence respiratoire,
* une augmentation de la vitesse du métabolisme,
* une modification de la circulation sanguine, menant à une vigilance accrue et à une activité réduite des systèmes digestif et urinaire.

Ces réponses au stress sont effectuées à court terme.

La réponse au stress à long terme est réalisée par les corticostéroïdes, libérés par le cortex surrénal (**axe corticotrope**).

L'activation de l'axe corticotrope débute par une stimulation de l'hypothalamus par le système limbique et l'amygdale. Cette stimulation conduit à la libération de corticolibérine (CRH) dans le système porte hypophysaire. La CRH est le principal régulateur de la sécrétion d'hormone adrénocorticotrope (ACTH) par l'adénohypophyse

(Chrousos & Gold, 1992). En réponse à l'ACTH, les cellules stéroïdogènes des glandes corticosurrénales utilisent le cholestérol pour produire des hormones stéroïdes. Parmi celles-ci, les glucocorticoïdes (**cortisol et corticostérone**) constituent les effecteurs finaux de la réponse neuroendocrine au stress chronique (Ottaviani & Franceschi, 1996). En effet, lorsqu'un animal fait face à un facteur de stress, les concentrations plasmatiques en cortisol (chez la plupart des Mammifères) et en corticostérone (chez les rongeurs notamment), augmentent plus ou moins rapidement en fonction de la nature, de l'intensité et de la durée de la perturbation. Les glucocorticoïdes entraînent :

* une dégradation de protéines, lipides, et une augmentation de la glycémie,
* une diminution de certains aspects de l'immunité,
* une rétention d'ions sodium et de l'eau par les reins,
* une augmentation du volume sanguin et de la pression artérielle.

L'activité des glucocorticoïdes est donc liée au métabolisme glucidique, lipidique et protéique. La sécrétion de glucocorticoïdes entraîne ainsi une sollicitation des réserves énergétiques et une hyperglycémie (Ottaviani & Franceschi, 1996). Il s'agit en fait d'une redistribution globale de l'énergie, de façon à faciliter l'adaptation de l'organisme. Cette redistribution se fait au détriment de certaines fonctions physiologiques n'étant pas immédiatement nécessaires. L'axe thyroïdien, et certaines fonctions immunitaires (fonctions pro-inflammatoires), se trouvent ainsi inhibées (Chrousos & Gold, 1992; Chrousos, 1998b). Cependant, certains types de stress stimulent la production des **hormones thyroïdiennes** (Fukuhara *et al.*, 1996), démontrant ainsi la pluralité et la grande spécificité de la réponse au stress. En résumé, les fonctions thyroïdiennes et immunitaires non immédiatement nécessaires sont temporairement inhibées de manière à effectuer une redistribution adaptative de l'énergie.

Les glucocorticoïdes exercent par ailleurs un rétrocontrôle négatif sur les éléments centraux de l'axe corticostérone, afin de protéger l'individu d'une réponse disproportionnée. En effet, lorsque la concentration en glucocorticoïdes reste élevée de manière chronique, on rentre dans une phase d'épuisement, et la réponse au stress perd ses propriétés adaptatives. On constate ainsi l'apparition de divers effets délétères

susceptibles d'être majorés au cours de la période de développement. Ainsi, la diminution de la sécrétion d'hormones thyroïdiennes peut inhiber la croissance musculaire, la croissance osseuse et la maturation du système nerveux central (Koibuchi & Iwasaki, 2006). La sécrétion prolongée de glucocorticoïdes peut également entraîner un épuisement des réserves énergétiques. On observe alors des problèmes de croissance liés parfois à des atrophies musculaires (Munk *et al.*, 1984; Hartmann *et al.*, 1999).

Par conséquent, une obstruction nasale chez un jeune, entraîne de nombreuses perturbations aussi bien au niveau morphologique, physiologique et comportemental.

*** Ces perturbations apparaissent-elles dès le 1er jour de l'obstruction nasale ? C'est à dire pendant la période d'obstruction nasale ? Ou plusieurs jours après la réouverture des narines ?**

*** Ces perturbations ont-elles des conséquences à long terme sur le développement morphologique, l'état physiologique et le comportement des individus ?**

*** De plus, si l'individu pendant sa période d'apprentissage de reconnaissance olfactives des congénères, est perturbé à cause d'une obstruction nasale, y aura-t-il à long terme, une répercution sur son comportement sexuel et ses réactions émotionnelles ?**

En 2007, dans le cadre de sa thèse au sein du laboratoire de Physiologie du Comportement de Nancy I, Mathieu Gelhaye a étudié les incidences comportementales et physiologiques d'une obstruction nasale bilatérale chez le rat en développement. L'objectif de son travail expérimental était de tester l'hypothèse selon laquelle une obstruction nasale bilatérale chez les jeunes, pouvait induire un stress chronique et perturber le comportement exploratoire nécessaire à la dispersion des jeunes après le sevrage. Pour tester son hypothèse, il a effectué une obstruction nasale chez des rats de 8 jours (J8) et il a observé les effets à la fin de la période d'obstruction (J15) et six jours après la réouverture des narines (J21). Les résultats obtenus par Gelhaye *et al.*, (2006b, 2007) montrent que l'obstruction nasale perturbe le développement des jeunes (retard de

croissance) et la mise en place du comportement exploratoire des individus. Ainsi, l'orientation par rapport aux odeurs familières, les réactions face à la nouveauté et les performances dans une tâche d'apprentissage spatiale, sont altérées à J21. De plus, les jeunes rats de 21 jours présentent une adaptation des muscles oro-faciaux (Gelhaye *et al.,* 2006a). Cette respiration buccale s'accompagne au niveau du système immunitaire, d'une baisse de la masse thymique et de la réponse proliférative des thymocytes chez les femelles à 21 jours. D'autre part, au niveau endocrinien, l'obstruction nasale entraîne une hyper-corticostéronémie et un hypothyroïdisme. Ces modifications hormonales sont globalement plus marquées chez les femelles. L'obstruction nasale constitue donc un facteur de stress chez les jeunes rats tout comme pour les jeunes enfants.

Dans le cadre de ce travail de thèse, nous avons repris le même protocole expérimental utilisé par Mathieu Gelhaye (2007) dans le cadre de sa thèse. Nous avons effectué une obstruction nasale bilatérale et réversible par cautérisation sur de jeunes rats de 8 jours (figure 4).

Figure 4 : Jeunes rats Wistar âgés de 8 jours avec une obstruction nasale bilatérale (expérimental) ou une brûlure superficielle (contrôle)

Mais dans le cadre de notre étude, nous avons focalisé nos observations sur les effets de cette obstruction pendant la période de fermeture complète des narines (J9 à J12), puis à long terme, soit 3 mois après leur réouverture (J90). Nous avons réalisé une étude pluridisciplinaire pour nous permettre d'aborder les

effets de cette obstruction nasale sur le développement morphologique, l'état physiologique et le comportement des jeunes pendant cette période de stress, et les répercussions possibles de ce stress à l'âge adulte.

Ce travail de thèse comprend 3 parties basées sur 5 publications scientifiques issues de notre travail de recherche:

Dans le ***chapitre I,*** *nous observons le comportement des mères face à leurs jeunes de 9 jours présentant une obstruction nasale, nous testons la capacité des jeunes à retrouver le nid maternel et nous effectuons une étude de l'état hormonal (corticostérone, hormones thyroïdiennes, vasopressine) et nutritionnel de ces jeunes pendant l'obstruction nasale (J9 à J15). Ce travail a donné lieu à deux publications :*

* *Article 1* : Gelhaye M., **Padzys G.S**., Orly J.C., Thornton S., Martrette J.M. & Trabalon M. 2011. Mother-Pupe interactions during a short olfactory deprivation period in young rats. *Development. Psychobiol.* **53**, 303-316.

* *Article 2* : **Padzys G.S**., Thornton S.N., Martrette J.M. & M. Trabalon. 2011. Effects of short term forced oral breathing in rat pups on weight gain, hydration and stress. *Physiol. Behav.***102**, 175-180.

Dans le ***chapitre II,*** *nous abordons le développement morphologique, en particulier l'adaptation morphologique des muscles oro-faciaux et du crâne, en relation avec l'état physiologique des individus pendant la période de l'obstruction nasale (J9 et J11), puis à l'âge adulte (J90). Ce travail a permis la rédaction de deux articles actuellement en révision mineure :*

* *Article 3* : **Padzys G.S**., Martrette J.M., Tankosic C., Thornton S.N. & M. Trabalon. 2011. Effects of short term forced oral breathing : physiological changes and structural adaptation of diaphragm and orofacial muscles in rats. *Arch. Oral Biol.*

* *Article 4* : **Padzys G.S.,** Tankosic C., Trabalon M. & J.M. Martrette. 2011 Craniofacial development and physiological state after early oral breathing in rat. *Eur. J. Oral Sci.*

*Dans le **chapitre III**, nous abordons l'étude préliminaire du comportement sexuel des rats **,** 90 jours après la réouverture des narines, en relation avec la capacité de détection olfactive et l'état physiologique (hormones sexuelles) des individus mâles. Les résultats obtenus sont soumis pour la publication suivante :*

* *Article 5* : **Padzys G.S.**, Martrette J.M., Thornton S.N. & M. Trabalon. Sexual differentiation of odor and physiological profile in adult male rats after a neonatal, short term, forced oral breathing period.

CHAPITRE I

Incidences d’une obstruction nasale précoce sur le comportement mère-jeunes et sur le développement des jeunes

Chez les Mammifères la mise en place de la relation mère - jeunes est une étape importante pour la survie et le développement du nouveau-né appartenant aux espèces nidicoles, comme les rats. Le rat naît au sein d'une portée nombreuse (10-12 jeunes), dans un état d'immaturité (figure 5) qui le rend complètement dépendant de sa mère dans les premiers stades de son développement (Denenberg *et al.*, 1962 ; Lee & Williams, 1977 ; Hofer, 1981).

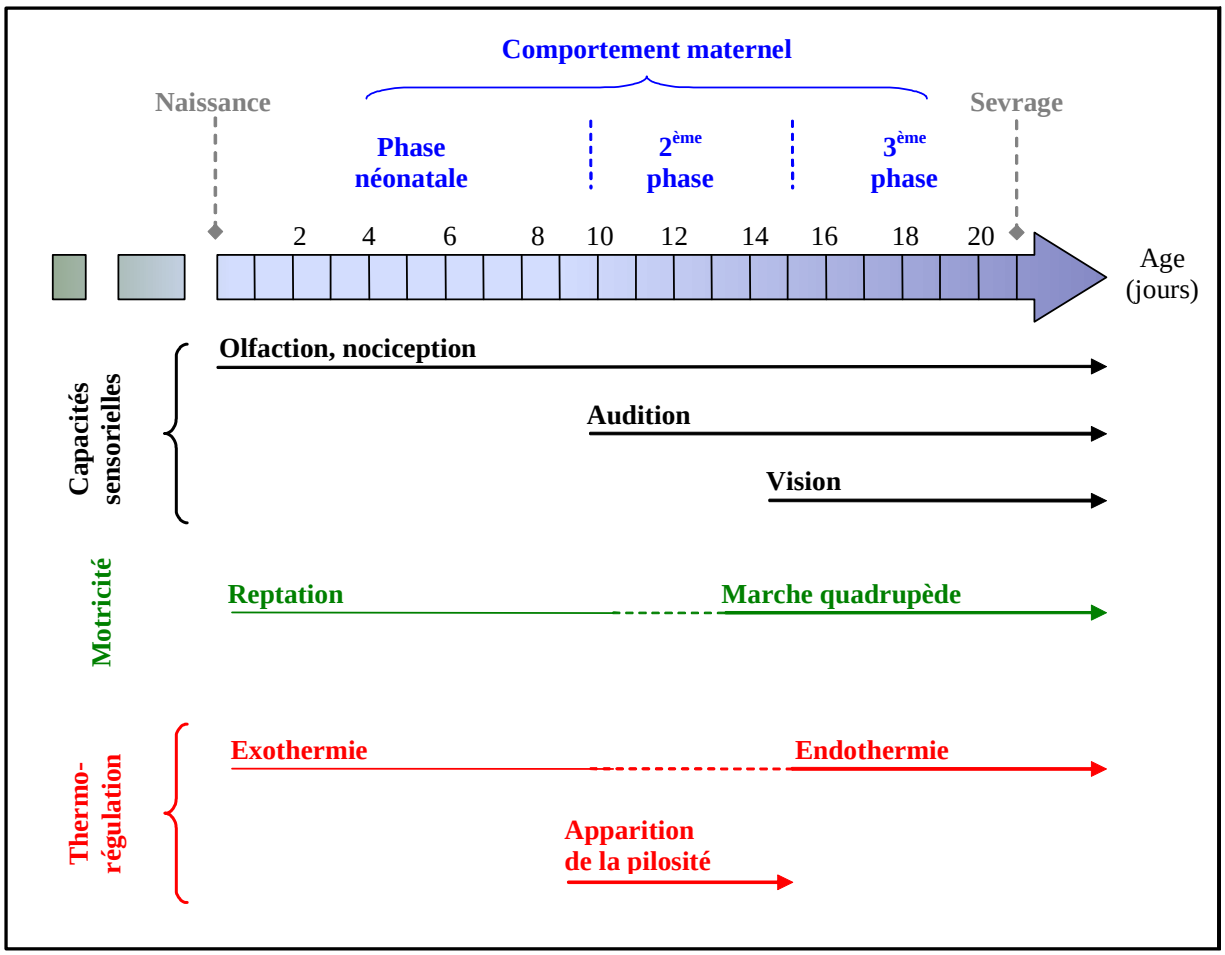

Figure 5 : Développement neurosensoriel du rat et évolution des relations mère – jeunes.

Les relations mère-jeunes peuvent être divisées en trois phases successives.

* Au cours de la phase néonatale (J0-J10), les activités du nouveau-né sont essentiellement alimentaires, les allaitements étant initiés par la mère.
* Au cours de la deuxième phase, les jeunes acquièrent l'audition (J10), la locomotion quadrupède (J12), la vision (J14) et l'endothermie (J15). Le comportement exploratoire évolue en parallèle.
* Au cours de la troisième phase, c'est principalement les jeunes qui initient les événements alimentaires. Ils commencent à ingurgiter de la nourriture solide à partir du 18ème jour post-natal et sont alors sevrés vers le 21ème jour post-natal. Le répertoire comportemental s'accroît de manière importante jusqu'à ressembler à celui des adultes, et entraîne la dispersion des jeunes vers le 28ème jour post-natal.

Toutes les expériences des premiers moments de la vie vont être déterminantes pour l'avenir du jeune. De nombreuses influences subies au cours de ces premiers moments vont agir en dehors même du processus de l'attachement mère-jeune (empreinte). Ainsi arrivé à l'âge adulte, le choix d'un partenaire sexuel n'est pas déterminé que par les qualités de ce partenaire, mais aussi par l'interaction sociale vécue pendant la petite enfance.

1 – Relations Mère – jeunes non sevrés

Le terme « comportement maternel » recouvre un ensemble de conduites très diversifiées. Chez la rate, les principaux actes spécifiques connus du comportement maternel, en dehors de la parturition sont :

* la capacité de construire un nid quelques jours avant la parturition,
* l'activité de **léchage** de la progéniture (« **liching** »),
* la position arquée que prend le corps de la rate lors de l'allaitement (« **nursing** »),
* la faculté de ramener au nid tout nouveau-né qui s'en éloigne (« **retrieving**»),
* la réaction de protection de la progéniture lors d'un danger (Beach & Jaynes, 1956 ; Noirot, 1966 ; Rosenblatt, 1967, 1969, Roth & Rosenblatt, 1967).

Le comportement de léchage commence environ 3 h après la naissance du jeune rat et il est maintenu pendant les trois premières semaines de la vie du jeune. La plus grande partie du temps total de la toilette (90%) est consacré au léchage de la région ano-génitale (Brouette-Lahlou, 1989 ; Brouette-Lahlou et al., 1991b). Ce comportement spécifique (figure 6) est lié au fait que les nouveaux-nés ne peuvent ni uriner ni déféquer seuls. La mère doit lécher la région ano-génitale de chaque petit pour provoquer chez celui-ci un réflexe spinal lui permettant de vidanger la vessie et le colon (Rosenblatt *et al.*, 1979 ; Moore, 1981).

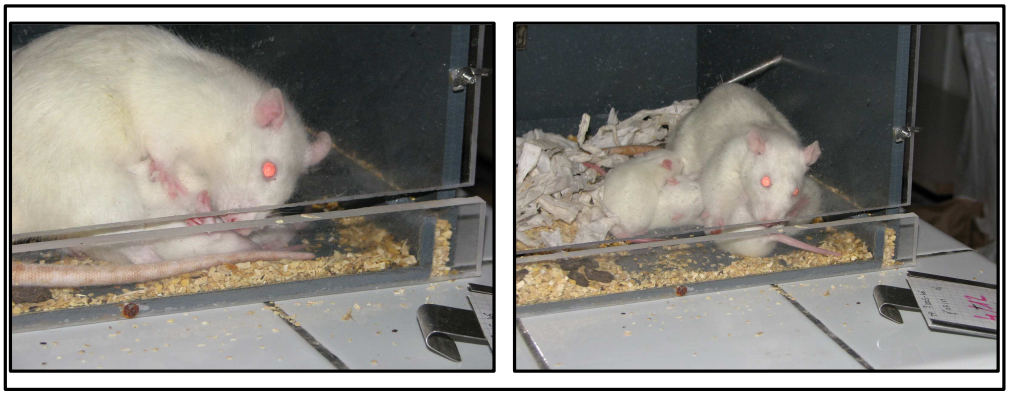

Figure 6: Léchage des régions anales et génitales externes.

L'ingestion d'eau et d'électrolytes de l'urine du jeune *via* le léchage ano-génital, permet de maintenir l'homéostasie des fluides corporels des jeunes (Gubernick et Alberts, 1983). Ce léchage peut affecter la composition du lait maternel, à mesure que les petits se développent (Moore, 1992).

Brouette-Lahlou *et al*., (1991b) ont montré que lorsque la région ano-génitale des ratons est nettoyée avec du savon, les mères ne lèchent que les têtes des jeunes. Ils ont également montré que le léchage ano-génital est également perturbé après l'ablation des glandes pré-putiales des jeunes : certains petits ne sont jamais léchés, ou ont été moins léché, tandis que d'autres ont été léché sans interruption, induisant une forte mortalité chez les nouveau-nés. L'utilisation de la chromatographie en phase gazeuse, couplée à la spectrométrie de masse, a permis de mettre en évidence un composé actif responsable de l'attraction ano-génitale des ratons : **le dodécyl propionate** (Brouette-Lahlou .*et al.*, 1991a, 1999). Le dodécyl propionate est un signal olfactif attractif qui aide la mère à

identifier les petits qui ont besoin d'être léchés. En ce sens, il régule mais ne déclenche pas le comportement de léchage ano-génital.

Moore (1992), a noté que les mères passent plus de temps à lécher les régions ano-génitales des mâles que celles des femelles. Si la mère est rendue temporairement anosmique, par un traitement chimique de l'épithélium olfactif, elle lèche moins ses petits, et ne distingue plus les mâles des femelles. Les mâles élevés par des mères anosmiques reçoivent moins de léchages ano-génitaux. La stimulation par la mère, de la région ano-génitale d'un mâle, permet la libération des hormones androgènes (testostérone) par les testicules du jeune mâle. Celles-ci sont nécessaires à la masculinisation du système nerveux central (aire préoptique médian de l'hypothalamus) et de la mœlle épinière (noyau spinal).

Ce comportement maternel vis-à-vis des jeunes mâles est un effet des androgènes eux-mêmes, car la mère détecte les jeunes mâles en flairant les métabolites des androgènes dans leur urine. D'autre part, cet effet est clairement le résultat d'une influence sociale : la mère traite différemment ses nourrissons mâles, masculinisant ainsi leur système nerveux en développement.

2 – Relations Jeunes non sevrés - Mère

La survie du jeune dépend de sa capacité à localiser la mamelle maternelle à téter. Au cours de la première semaine de vie, les jeunes rats sont sous contrôle d'un système intégratif qui contribue à les garder éveillés et motivés, puis à les guider vers les tétines pour qu'ils s'agrippent et tètent. Pendant cette période, la prise de lait n'est limitée que par la capacité gastrique du jeune (Hall & Rosenblatt, 1977). A partir du 14ème jour, la privation de lait entraîne des comportements de recherche active des mamelles (Hall *et al.*, 1975), ce qui laisse penser que l'attraction des tétines est de plus en plus contrôlée par des facteurs internes liés à l'état nutritif du jeune (Hall & Williams, 1983).

Chez les rats, l'orientation vers la mamelle et la prise de lait, sont dans un premier temps, contrôlés par des stimuli externes émis par la mère. Ces facteurs externes jouent un rôle primordial pendant la période post-natale. A partir de la deuxième semaine de vie, les facteurs internes (état physiologique du jeune) se mettent en place et deviennent

progressivement prépondérants dans la régulation des réponses du jeune aux odeurs maternelles.

De nombreuses expériences ont été réalisées chez des espèces de Mammifères pour étudier le rôle de l'olfaction chez le nouveau-né lors de la tétée.

* Chez le porcelet, les odeurs émises par la mère ont une action prépondérante dans la recherche et la prise en bouche d'une tétine. Morrow-Tesch & McGlone (1990a, b) ont montré que les signaux olfactifs maternels sont véhiculés par des fluides prénataux (liquide amniotique) et postnataux (lait, urine et fèces maternels).

* Chez le chat, la lésion des bulbes olfactifs supprime la capacité du jeune à trouver la mamelle et à téter (Kovach & Kling, 1967).

* Chez le rat, Terry & Johanson (1996) ont montré qu'à la naissance, les jeunes rats développent une attirance pour des odeurs lorsqu'elles sont associées à des stimuli de renforcement positif comme les soins maternels (toilettage, léchage ano-génital, ingestion de lait). Le conditionnement olfactif du jeune favorise ainsi l'activité motrice vers la mamelle, l'apparition d'un comportement de vigilance et les premiers apprentissages comme la sélectivité alimentaire (Nowak *et al.*, 2000; Coureaud *et al.*, 2001). La bulbectomie olfactive bilatérale, effectuée sur de jeunes rongeurs, entraîne des difficultés d'orientation vers les mamelles, une diminution du taux d'attachement aux mamelles et un retard de croissance (Singh & Tobach, 1975; Risser & Slotnick, 1987). D'autres expériences ont montré que les jeunes rats sont incapables de se diriger vers les mamelles maternelles après un lavage préalable avec des solvants organiques. A l'aide d' essais de badigeonnages de diverses substances odorantes sur les mamelles lavées, Teicher & Blass (1976, 1977) et Blass & Teicher (1980) ont montré que la mère émet trois principaux substrats chimiques qui permettent d'orienter le jeune vers les mamelles et d'entrainer la tétée. Ces trois composés chimiques sont présents dans le liquide amniotique, la salive de la mère et du jeune. Au cours de la première semaine de vie, les jeunes rats réagissent d'abord à l'odeur du liquide amniotique, puis à l'odeur de la salive maternelle et juvénile, et enfin à l'odeur du lait (Blass & Teicher, 1980). Brouette-Lahlou, 1991a a montré que le **diméthyl-disulfite,** libéré par la mère rate**,** entraîne l'orientation des jeunes nouveaux-nés vers la mamelle et le déclenchement de la première tétée. Initialement, ce composé est présent dans le liquide amniotique de la mère. Il est ingéré par la mère au moment de la mise bas, puis déposé par la mère lors des léchages de ses mamelles. Après la première tétée, le composé se retrouve dans la

salive du jeune, qui le dépose à nouveau sur les mamelles, maintenant ainsi leur attractivité pendant la période d'allaitement.

* Chez d'autres espèces de Mammifères l'orientation vers les mamelles se fait grâce à des signaux chimiques émit directement au niveau de la région mammaire (Pedersen & Blass 1982 ; Pedersen *et al.,* 1982 ; Morrow-Tesch et McGlone 1990a; Porter *et al*., 1991).

* Chez le lapin, c'est le **méthylbut-2-ène** synthétisé au niveau de la glande mammaire, qui suscite le comportement de recherche mammaire chez le jeune (Schaal *et al.,* 2003).

En cas d'obstruction nasale, la compétition entre les processus respiratoire et alimentaire pourrait constituer un facteur renforçant les impacts de la privation olfactive sur l'alimentation. Cette compétition pourrait notamment perturber la saisie orale des mamelles et limiter le rythme d'ingestion du lait maternel. On sait que chez les Mammifères, une privation de lait maternel de courte durée, induit des perturbations endocriniennes, telles qu'une inhibition de l'axe thyroïdien et une hypercorticostéronémie (Oberkotter & Rasmussen 1992; Schmidt *et al.,* 2002). En jouant sur la prise alimentaire, l'hyposmie associée à l'obstruction nasale pourrait donc perturber de manière importante l'homéostasie hormonale du jeune en développement.

Par ailleurs, l'olfaction joue un rôle primordial dans les relations qu'entretiennent les jeunes avec leur environnement social et physique. La détection des odeurs permet notamment la mise en place des mécanismes d'orientation, d'attraction ou d'évitement, en fonction des caractéristiques du stimulus.

L'apprentissage des odeurs familières chez les Mammifères débute *in utero via* le liquide amniotique (Schaal et Orgeur, 1992), puis l'acquisition et le stockage des informations olfactives se poursuivent à la naissance. Ainsi, les mères lactantes de plusieurs espèces de rongeurs émettent des signaux chimiques attractifs uniquement pour leurs jeunes qui s'orientent préférentiellement vers les sites saturés de l'odeur maternelle (Leon & Moltz, 1971). Le nid maternel est donc imprégné d'odeurs qui délimitent le territoire des jeunes, restreignent et orientent leurs déplacements. Chez le rat, l'odeur du nid représente un stimulus attractif dès le troisième jour postnatal (Cornwell-Jones & Sobrian, 1977) ; le jeune présente alors des préférences olfactives

pour son propre nid par rapport à un nid étranger (Brown, 1982). Les jeunes rats préfèrent toutefois s'orienter vers l'odeur de leur mère que vers l'odeur de leur nid (Polan & Hofer, 1998). Les signaux olfactifs induisent également des préférences filiales pour le regroupement des ratons au sein du nid (Brunjes & Alberts, 1979). Cette préférence est supprimée par l'anosmie chimique au sulfate de zinc bien que le regroupement ne soit pas éliminé pour autant (Alberts & Brunjes, 1978). Par ailleurs l'apprentissage olfactif initié depuis la vie utérine, et qui se poursuit jusqu'au sevrage, va permettre la mise en place de la **mémoire sociale olfactive**. Elle va jouer un rôle primordial dans le choix du partenaire de jeux, puis du partenaire sexuel, en fonction de l'odeur apprise durant la période post-natale et durant l'enfance (Loranca & Salas 2001 ; Bakker *et al*., 1996; Gasperin-Estrada *et al.,* 2008).

Chez le rat, les signaux olfactifs présents dans les crottes maternelles jouent également un rôle essentiel dans le développement physiologique du jeune. Ainsi, certains métabolites de l'acide cholique, tels que l'acide désoxycholique sont présents dans les cæcotrophes maternels et sont consommé par le jeune à partir du $13^{ème}$ - $14^{ème}$ jour postnatal (Kilpatrick *et al.,* 1980). Ce composé agit sur l'immunocompétence et la myélinisation du système nerveux. En effet, les ratons privés des cæcotrophes montrent un taux de mortalité élevé associé à un retard de croissance cérébrale et de développement neuro - comportemental.

3 – Incidences d'une obstruction nasale sur les relations mère - jeunes.

Nos résultats montrent que les mères des portées exposées à l'obstruction nasale mettent moins de temps à ramener les jeunes dans le nid et passent plus de temps à lécher leur progéniture (cf. article 1). Ces modifications nécessitent sans doute le traitement simultané de signaux visuels, acoustiques et olfactifs.

Il est bien établi que les jeunes rongeurs en situation de détresse émettent des vocalisations ultrasoniques (Hofer & Shair, 1992). Il a notamment été montré que le comportement de " retrieving " est lié à ces vocalisations émises par la progéniture

éloignée du nid (Hahn & Lavooy, 2005). Les appels ultrasoniques d'isolation initient ainsi les comportements de recherche et de transport par la mère. Ils raccourcissent en outre la latence de récupération des jeunes (Brewster & Leon, 1980). De plus, le nombre d'appels est susceptible de modifier le comportement de la mère (Brudzynski, 2005). Étant donné qu'un niveau élevé d'anxiété entraîne une augmentation du taux de vocalisations (Naito et al., 2000), un nombre d'appels plus élevé pourrait expliquer la réduction du temps mis pour ramener les jeunes au sein du nid.

Les vocalisations ultrasoniques contribuent également à accroître le temps de léchage ano-génital suite à la récupération des jeunes (Brouette-Lahlou et al., 1992), ce qui est en accord avec les résultats (cf. article 1*)*.

En plus de l'influence des signaux acoustiques, le léchage ano-génital est fortement dépendant des signaux chimiques présents dans l'urine des jeunes (Brouette-Lahlou et al., 1999). Ainsi, les mères pourraient percevoir les impacts de l'obstruction nasale au travers de la détection de signaux chimiques présents dans l'urine de la progéniture.

D'autres études ont montré l'influence des expériences olfactives précoces sur le développement neuro-comportemental des rongeurs (Sczerzenie & Hsiao, 1977; Coopersmith & Leon, 1984 ; Hongo et al., 2000). La mère constituant l'unique source de nourriture, de chaleur et de protection, l'apprentissage de l'odeur maternelle est vitale pour le jeune (Sullivan, 2003). Le nouveau-né doit rapidement apprendre l'odeur de sa mère, puis de son nid et de ses congénères, afin d'orienter ses déplacements. En privant le jeune des signaux olfactifs en provenance de sa mère et de son environnement biotique (congénères) et abiotiques (nid), l'obstruction nasale bilatérale est susceptible d'entraîner un isolement social partiel et donc un stress sensoriel. En effet, du fait de leur développement cérébral postnatal, les ratons ne disposent que de l'olfaction pour s'orienter à distance. L'audition et la vision n'étant respectivement acquises qu'à partir du dixième et du quatorzième jour postnatal, les individus exposés à l'obstruction nasale ne disposent que des signaux tactiles et thermiques pour faire face à l'appauvrissement global de leur environnement olfactif.

Nos résultats montrent qu'à 9 jours, l'odeur du nid constitue un stimulus attractif susceptible d'induire une activité locomotrice chez les jeunes contrôles et témoins (cf. article 1). Mais ils montrent également que l'obstruction nasale précoce a un impact fonctionnel sur les capacités olfactives qui perturbe l'orientation vis-à-vis du nid 24 h après le traitement (J9). En effet, les animaux exposés à l'obstruction nasale présentent un taux de retour au nid plus faible et distribuent leur temps de manière aléatoire dans les trois branches du labyrinthe. Ces animaux réalisent significativement moins de choix et affichent un temps de latence élevé comparés aux animaux témoins et contrôles, ce qui suggère une perturbation du comportement exploratoire d'investigation. Ces données pourraient être expliquées non seulement par la privation olfactive, mais également par l'accroissement du niveau d'anxiété (révélé par des taux plasmatiques élevés de corticostérone et faibles d'hormones thyroïdiennes) qui pourrait agir sur l'investigation de l'environnement.

En terme de récupération juste après la réouverture des narines (J15), le taux de retour au nid et le temps passé du côté du nid restent significativement différents mais les jeunes expérimentaux explorent autant que les rats contrôles et témoins. Ceci suggère un rétablissement rapide du comportement exploratoire, mais une récupération plus lente des capacités olfactives en dépit de la réouverture des narines. Le prompt rétablissement du comportement exploratoire pourrait être expliqué par le fait qu'avec l'apparition de l'audition à J10 et de la vision à J14, l'importance relative de l'olfaction dans l'expression du comportement exploratoire diminue progressivement au cours du développement.

4 - Incidences d'une obstruction nasale sur le développement des jeunes

Mathieu Gelhaye (2007), a montré que les animaux, exposés à l'obstruction nasale, présentent un taux de mortalité élevé qui survient essentiellement à J11, c'est-à-dire 72h après la cautérisation des narines. Le taux de mortalité augmente progressivement jusqu'à J14 avant de se stabiliser autour de 37 % à partir du moment où les narines commencent à s'ouvrir. Ce taux de mortalité important ne provient pas d'une inhibition du comportement maternel puisque nos résultats montrent que la mère augmente son activité de soin aux jeunes qui présentent une obstruction nasale.

Nous avons mesuré, à l'aide d'un respiromètre – oxymètre (Fisher Scientific, France), la quantité moyenne d'oxygène consommé par nos rats ainsi que la quantité moyenne de gaz carbonique rejeté pendant la période d'obstruction nasale (Figure 7 ; résultats non publiés). Nos résultats montrent que la consommation d'oxygène effectuée par les jeunes rats expérimentaux, pendant toute la période de l'obstruction nasale, est significativement inférieure à celle des rats contrôles. La quantité de CO_2 rejeté est significativement inférieure chez les femelles expérimentales âgées de 11 jours et chez les mâles de 10 et 11 jours. La quantité de CO_2 plus faible chez les rats expérimentaux semble être liée à une activité motrice plus faible de ces rats dans l'enceinte expérimentale par rapport aux rats contrôles.

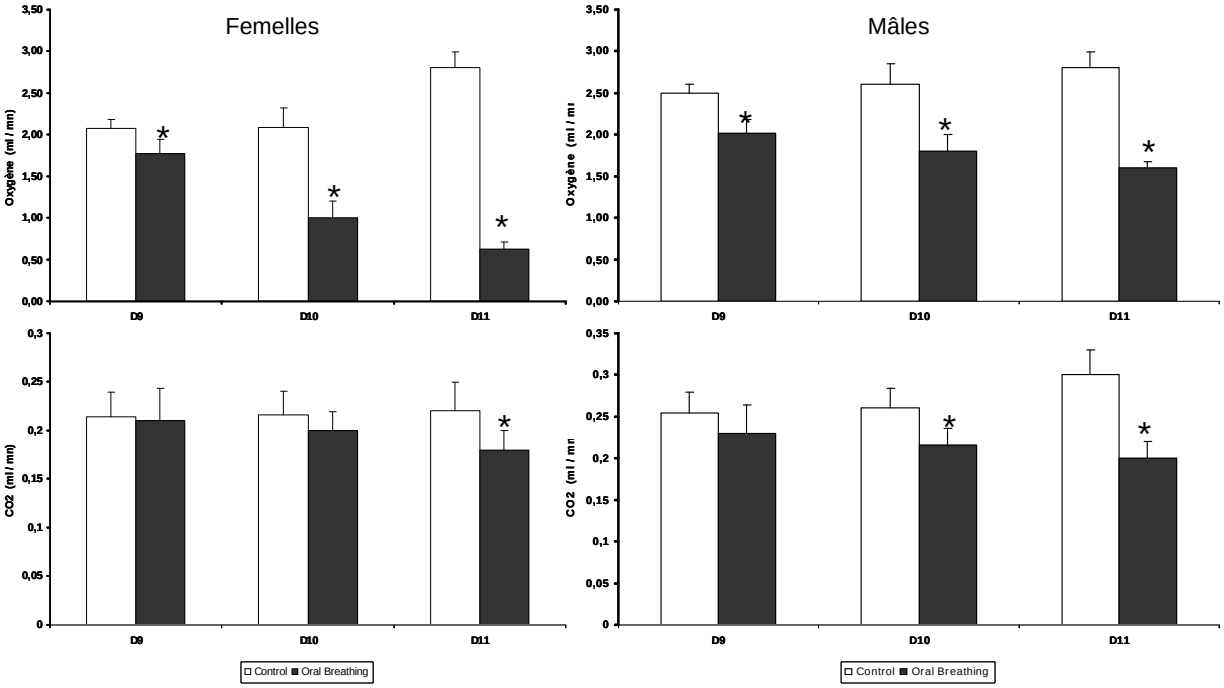

Figure 7 : Quantité d'oxygène consommé et de gaz carbonique rejeté (en ml / ml) par les rats expérimentaux (Oral breathing) et les rats contrôles pendant la période d'obstruction nasale (D9, D10 et D11). Les résultats sont exprimés en moyenne ± SE. * significativement différent des contrôles à P = 0.05.

Cette détresse respiratoire, induite par l'obstruction nasale est sans doute partiellement responsable de l'important taux de mortalité observé. En effet, le rat présente une forte résistance des voies aériennes oro-pharyngées. Or en conditions physiologiques normales, la ventilation s'effectue par la voie nasale, éventuellement suppléé par la bouche lors d'efforts physiques ou d'encombrement temporaire des voies nasales (Pohunek, 2004). Au niveau des cavités nasales, le débit aérien est régulé par les

valvules narinaires contrôlées par les muscles alaires qui réalisent des mouvements symétriques et synchronisés avec les mouvements respiratoires (Cole, 2003). D'autre part, l'alternance de surfaces concaves et convexes freine l'air en provoquant la formation de turbulences. Cet écoulement turbulent jouerait un rôle essentiel dans la croissance des cavités nasales (Churchill *et al.*, 2004). Le ralentissement du flux aérien permet en outre d'améliorer le conditionnement de l'air inspiré (Churchill *et al.*, 2004). Les cavités nasales assurent en effet le conditionnement de l'air de façon à optimiser les échanges gazeux dans les poumons (Ingelstedt, 1956). Elles possèdent ainsi la capacité de chauffer et d'humidifier l'air inhalé, ce conditionnement s'effectue particulièrement dans le segment nasal antérieur qui possède les caractéristiques anatomiques nécessaires (Keck *et al.*, 2001).

L'absence de ventilation nasale a de nombreuses conséquences sur les plans local et régional :

* assèchement des muqueuses nasale et buccale (Svensson *et al.*, 2006),
* accumulation de CO_2 dans les sinus paranasaux (Ganjian *et al.*, 1999),
* altération du drainage mucociliaire (Raji *et al.*, 2001),
* manifestations otologiques, ophtalmologiques et morphométriques délétères (Buchman *et al.*, 1999; Gola *et al.*, 2002; Shikata *et al.*, 2004).

Cette transition a également des conséquences importantes sur le plan général. En effet, non seulement la respiration buccale forcée pourrait perturber l'alimentation du fait de la compétition entre les processus respiratoire et alimentaire (Erkan *et al.*, 1994), mais elle influe également sur l'homéostasie gazeuse du sang et sur l'activité électromyographique des muscles or-faciaux. L'obstruction nasale se traduit ainsi par une hypoxie, une hypercapnie et une acidémie (Harding *et al.*, 1987; Tacx et Strack Van Schijndel, 2003). Les études chez l'animal montrent qu'en cas d'obstruction nasale, les perturbations de l'homéostasie gazeuse du sang sont plus marquées chez le nouveau-né (Harding & Wood, 1990). Ces modifications ont été observées à des degrés divers chez le rat (Erkan *et al.*, 1994), le lapin (Ramadan, 1983), la brebis (Harding *et al.*, 1991), le chien (Cavo *et al.*, 1975) et l'être humain (Cvetnic *et al.*, 1981). Chez les animaux à respiration nasale obligatoire comme le rat, cette insuffisance respiratoire est suceptible d'être renforcée par la forte résistance des voies aériennes oro-pharyngées (Kalogjera *et*

al., 1991). Chez le rat adulte, Erkan *et al.*, (1994) rapportent une diminution du pH et de la pression partielle en O_2 s'aggravant au cours des 72h suivant l'occlusion des narines. Ces modifications de l'homéostasie gazeuse du sang seraient à l'origine de la mort des animaux entre 90 et 100 h après l'induction de l'obstruction nasale. Ainsi, Nakajima & Ohi (1977) ont montré que les animaux décédés suite à l'induction d'une obstruction nasale expérimentale, présentaient des nécroses et des hémorragies au niveau de l'intestin grêle. Ces modifications sont liées à une accumulation excessive de gaz dans le tractus gastro-intestinal des individus. En effet, au moment de l'alimentation, l'obstruction nasale entraîne une compétition entre les processus respiratoire et alimentaire se traduisant par une aérophagie. Selon Kalogjera *et al.,* (1991), le décès serait lié à cette aérophagie qui provoquerait une élévation du diaphragme et un iléus paralytique (occlusion intestinale due à une paralysie de l'intestin grêle). Ce dernier entraînerait un arrêt du transit intestinal. De plus, l'aérophagie néonatale peut être à l'origine d'une perforation gastrique létale comme cela a été préalablement montré chez le rat, le chien et l'être humain (Shaker *et al.*, 1973; Leone & Krasna, 2000). L'obstruction nasale perturbant la prise alimentaire, une forte réduction de l'apport énergétique pourrait donc être également impliquée dans la mortalité élevée des animaux .

*Dans ce cadre, nos résultats montrent effectivement que pendant l'obstruction nasale (J9 à J15), la croissance pondérale des animaux expérimentaux est significativement plus faible que chez les animaux contrôles (**cf. article 1 et 2**). La prise de nourriture (lait maternel) ne s'effectue pas de manière régulière, ce qui entraîne une rapide déshydratation et une baisse de la glycémie chez les jeunes expérimentaux. Cette déshydratation est révélée par une augmentation de l'osmolarité, et une libération importante de la vasopressine, hormone responsable de la régulation des pertes hydriques du corps. Après la réouverture des narines, le jeune peut à nouveau s'alimenter normalement et la déshydratation disparaît, mais les taux plasmatiques de la corticostérone restent élevés ce qui laisse suggérer que les jeunes ont un niveau de stress supérieur aux jeunes contrôles.*

Ces résultats sont semblables à ceux observés chez l'homme où le passage à la respiration orale est associé à une **perte hydrique** importante, dûe à un assèchement de la muqueuse buccale. En effet quelques heures après la mise en place de la respiration

orale on observe une **déshydratation** associée à une perte énergétique (Svensson *et al.*, 2006).

Les modifications respiratoires peuvent également être à l'origine des troubles du sommeil. L'obstruction des voies aériennes supérieures entraîne des efforts respiratoires pouvant induire des micro-éveils et ainsi fractionner le sommeil. Ce phénomène, appelé syndrome d'apnées obstructives du sommeil, est caractérisé par un arrêt du flux aérien d'une durée supérieure ou égale à 10 secondes, la reprise respiratoire coïncidant habituellement avec un éveil très bref ou avec l'allégement du sommeil (Zwillich *et al.*, 1981).

Article 1

Mother-pup interactions during a short olfactory deprivation period in young rats

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***Developmental Psychobiology*, 2011, 53, 303-315**

1 - Introduction

Several ontogenic studies have demonstrated the importance of early olfactory experience in the neurobehavioral development of rodents (Coopersmith & Leon, 1984; Miller & Spear, 2010). In the rat neonate, olfactory cues from the mother and siblings are crucial in the establishment of early behaviours such as nursing (Hongo *et al.*, 2000), huddling (Brunjes & Alberts, 1979) and home orientation (Sczerzenie & Hsiao, 1977). Newborn altricial mammals must learn the odor of their mother and use it to orient their displacements. As the mother is the sole source of food, warmth and protection, learning this odor is critical for the survival of the newborn (Sullivan, 2003). For these different reasons, a chronic olfactory deficit can have significant consequence on the homeostasis of the young individual. In the last decade, many experimental studies were carried out requiring the induction of an early olfactory deprivation. A common technique employed to produce transient olfactory deprivation in neonatal animals consists in the obstruction of the nasal cavities (Meisami, 1976; Waguespack *et al.*, 2005). This short term procedure (lasting from 4 to 6 days) generates numerous long term effects on the olfactory bulb, including reduction of its volume and a variety of physiological and biochemical alterations in the pup (Brunjes *et al.*, 1985; Brunjes, 1994). However, the functional impact of bilateral naris occlusion on olfactory ability has never been investigated.

Our previous studies revealed that bilateral naris occlusion in eight-day old rat pups was associated with an impairment of novelty-seeking behaviour, an adrenal hypertrophy and an increase in circulating glucocorticoids at 21 days of age (Gelhaye *et al.*, 2006b). These modifications were associated with a decrease of splenocyte and

thymocyte proliferative responses and with an adaptation of orofacial muscles, facilitating respiration (Gelhaye *et al.*, 2006a, 2006b). Consequently, we concluded that early bilateral naris occlusion could be regarded as a multi- factorial stressful situation, which ultimately affected peripheral physiological systems. These effects could be explained, at least partially, by a disturbance of mother-pup interactions, which had an effect on endocrine balance. In rodents, there is indeed a reciprocal regulation of responsiveness to stress between mother and offspring (Walker et al., 2004). For example, dietary influences are critical in the modulation of the stress response and it was shown that olfactory signals were essential in the orientation to the nipples in several species of rodents (Blass & Teicher, 1980; Gerling & Yahr, 1982; Coureaud & Schaal, 2000). By reducing olfactory abilities, early bilareral naris occlusion could thus have important functional repercussions on mother-pup interaction and notably on the offspring's suckling behaviour.

It is well known that both the quantity and the quality of the ingested food play a crucial part in the maintenance of hormonal homeostasis during the postnatal period. Mother-milk deprivation of a few hours was shown to produce a significant reduction in thyroxin levels and an increase of plasma corticosterone levels (Oberkotter, 1988; Schmidt et al., 2002). Both thyroid hormones and corticosterone play a key role in normal development of mammals. Neonatal thyroid hormone deficiency disturbs brain development, suppresses the proliferation of lymphocyte B precursors and delays the maturation of orofacial muscles (Arpin et al., 2000; Koibuchi & Chin, 2000; Ganji & Behzadi, 2007). For that reason, the fundamental assumption of the experimental work presented in this study is that bilateral naris occlusion produces olfactory deprivation which has effects on peripheral physiological systems that could be linked, at least partially, to a disturbance of mother-pup interactions, which would generate alterations in the corticotrope and thyroid axes.

Consequently, the aim of the present study was to evaluate the effect of early short olfactory deprivation on mother-pup interactions during early post-natal development in rats. We performed olfactory deprived in eight-day old rats and studied its effects on behaviour at the beginning of postnatal day 9 (PND 9) and at the end of the olfactory deprived period i.e. postnatal day 15 (PND 15).

Our hypothesis was that early short term olfactory deprivation would have a significant effect of some parameters of mother-pup interactions, focusing more particularly on suckling behaviour. We investigated also the functional impact of early

olfactory deprivation on olfactory abilities. Nest recognition was therefore investigated in a two-choice situation. The effects of early olfactory deprivation on the stress response and on plasma levels of thyroid hormones (T3 and T4) was also studied in 9 and 15-day old rats of both sexes.

2 – Methods

2.1. Experimental subjects

Four hundred and fifty pups from forty five mothers (Wistar, IFFA-CREDO) from post-natal day 8 (PND 8) to PND 15 were used. The animals were born in the laboratory and housed in standard cages under controlled temperature conditions (22 ± 1°C). Food and water were available *ad libitum* during the whole experiment. From birth, the litters were kept on a reversed 12:12 light-dark cycle (dark period 08.00 hr – 20.00 hr) and were culled to five males and five females to ensure normal body growth (10 rats per litter before the culling procedure).

2.2. Bilateral naris occlusion procedure

All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (n° 85-23, revised 1996) and the recommendations edited by the European Community Council for the Ethical Treatment of Animals (n°86/609/EEC).

At PND 8, the litters were randomly divided into three experimental groups. The untreated group was defined by the complete absence of manipulation. The sham group and animals with bilateral naris occlusion were first anesthetized by hypothermia (10min at -18°C). Once the litters were anesthetized, bilateral naris occlusion (inducing olfactory deprivation) was performed by cauterization of the external nostrils (Meisami, 1976; Waguespack et al., 2005). This is the most common and simple procedure allowing reversible naris occlusion in growing animals. The tissue surrounding the nostrils was burned by placing a surgical cauterizing instrument on the nostrils, consequently occluding the orifice of the nostrils. In the days following the treatment, the reopening of the nostrils was assessed by applying a soapy solution; the absence of bubbles was used as an indicator of complete occlusion. This procedure induced olfactory deprivation between post natal day (PND) 8 and PND 12 with 93% of the nostrils spontaneously reopened at PND 14. Consequently, the different experiments were conducted at PND 9, i.e. 24 hr after the induction of the olfactory deprivation, and

at PND 15, age corresponding to the end of the olfactory deprivation period. In the sham group, the nostrils were not sealed but the tissue above them was burned by placing the cauterizing instrument about 1-2 mm above each nostril. After the cauterization, the burn was washed with chlortetracyclin (Aureomycin Evans 3%) to prevent possible infection. Sham and olfactory deprived animals were kept warm (37°C) for half an hour and then returned to their mothers. Sham and olfactory deprived pups were thus exposed to a single maternal separation of 45 min (10 min of hypothermia, 5 min for the cauterization, and 30 min of warming).

In order to avoid interference between the different experiments, the litters were randomly divided into animals used to test the mother-pup interactions (n = 5 mothers per group), animals used to test the growth parameters and the olfactory abilities (n = 15 rats per sex and per group) and animals used for the gastric content and hormonal assays (n = 8 rats per sex, per age and per group). We used one male and one female from each litter for each of the tests. For this we needed 45 litters.

2.3. Mother-pup interactions

All behavioural observations were made during the dark phase between 0900 hr and 1200 hr. The animals were videotaped with video tracking and SMART-MA logiciel version (Smart Panlab, Bioseb – France). An infrared camera was used during behavioural testing without the experimenter present in the room. The tapes were analysed at the end of all the recordings.

The different apparatus were maintained in the same position in the room throughout the duration of the study During the last week of gestation, the mothers were placed in home cages (65x32x50cm) conceived to facilitate behavioural observations. The front wall was made of transparent Plexiglas and the three other walls were made in gray PVC. The floor of the cage (0.2 m²) was covered by 100 ± 10 g of clean sawdust. Food and water were available *ad libitum* during the whole experiment. Five mothers per group were used to test the maternal and suckling behaviours. Maternal behaviour was only examined on PND 9. Indeed, with the neurobehavioral maturation of offspring (emergence of hearing, quadruped walkvision, thermoregulation, autonomous urination…), the expression of maternal behaviour gradually decreased during the postnatal period.

At PND 9 and 15, the mother was removed from the home cage for a period of 2 hr (0900 hr-1100 hr) before starting the behavioural observations (Stern & Johnson,

1990; Stern & Azzara, 2002). This maternal deprivation was performed in order to exacerbate maternal behaviour and enticement for the nipples. At 1100hr, the litters were temporarily culled to six rats (three females and three males) to avoid competition and to facilitate access to the nipples (Arrati et al., 2005). The removed animals were maintained in the presence of nest sawdust and their anogenital region was stroked periodically to limit the effects of additional maternal deprivation (van Oers et al., 1999). The animals used for the behavioural analysis were placed in the opposite side from the site where the mother built the nest. The mother was then returned to the home cage.

During the next 60mn (1100 hr – 1200 hr), the following behavioural items were recorded: duration of pup-retrieval (when the mother picks up the pups in her mouth and places them in the nest), duration of pup-licking (when the mother licks the body and/or the anogenital region of the pups) and duration of presence in the nest (when the mother is in the nest, her body over all or most of the pups) only on PND 9. In addition, the latency to nurse (when the mother adopts a nursing posture with at least one pup attached to the nipples), the duration of nursing and the maximum rate of nipple attachment (maximum number of simultaneously suckling rats) were recorded at PND 9 and PND 15.

2.4. Two-choice situation: return to the nest

In order to evaluate their olfactory ability, the rat pups were observed at PND 9 and PND 15 in the two-choice situation "nest sawdust *versus* clean sawdust". This test was performed in a T-maze with a start arm connected to two goal arms of equal dimensions. The maze was constructed of Plexiglas, with a guillotine door separating the start box from the main stem of the maze. An experimental box (30x18x14cm) was situated at the end of each goal arm and closed by a door with holes in it which allowed stimulation by smell. Two arm sizes were used in order to adapt the test to the rat's growth (PND 9:10x6x6cm; PND 15:25x8x8cm). In all cases, 45 ± 5 g of sawdust were placed in the experimental boxes and the nest sawdust was randomly placed in the right or left experimental box. The observation period (3 min) began when the rat entered the start arm of the T- maze and the guillotine door was closed behind it.

The percentage of rats carrying out a choice, the latency of the first choice (defined by the first contact between an animal and a lateral box), the side of the first

choice and the time spent in each arm of the T-maze were recorded. After the behavioural observations, animals were weighed and their length was measured from the nose to the base of the tail. The ponderal and longitudinal gains of each individual were calculated over the period PND 9 – PND 15.

3.5. Sample collection

At PND 9 or PND 15, immediately following sacrifice, intra-cardiac blood samplings (500-1000 µl) were performed between 1100 hr and 1200 hr for corticosterone, thyroxin and triiodothyronin measurements. Blood was collected within 1-2 min into sterile heparinised syringes. Plasma was immediately separated by centrifugation at 4°C (15 min at 3000 rpm) and the extracts were stored at -18°C until the time of assay. After blood sampling, gastric content, which constitutes a reliable index of the quantity of ingested milk (Fukushima et al., 2004), was removed and weighed.

3.6. Hormonal analysis

In order to assess the adrenal response after the induction of early nasal obstruction, corticosterone concentration was measured without an extraction procedure, using a commercially available EIA kit and according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentration of corticosterone in plasma samples was calculated from a standard curve and expressed as ng/ml. The intra- and inter-assay coefficients of variation were under 8.4 and 13.1 %, respectively. T4 and T3 were assayed using commercial RIA kits according to the manufacturer's guidelines (Immunotech SA, France). The concentrations of T4 and T3 in plasma samples were calculated from standard curves and expressed as pg/ml. The intra- and inter-assay coefficients of variation were respectively under 6.7 and 6.5% for T4 and under 6.4 and 5.5 % for T3.

3.7. Statistical Analyses

Data were expressed as group means ± SE. Statistical analysis was performed by means of statistical software (Statview V5.0, Abacus concepts Inc., Berkeley, CA). Concerning the behavioural data, the mother-pup interactions were examined using the Kruskal-Wallis analysis of variance because the samples were small and off a Gaussian distribution. The U-test of Mann-Whitney was used to establish the inter-group comparison. The two-choice situation was analyzed with the chi-square's test for the

percentage of rats carrying out a choice and the side of the first choice or with a two-way analysis of variance (sex * treatment) for the latency of the first choice and the time spent in each arm of the T-maze. In this last case, the PLSD Fisher procedure was used to establish the inter-group comparison.

Concerning the physiological data, after a two-way analysis of variance (sex * treatment), the PLSD Fisher procedure was used to establish the inter-group comparison. In all cases, the differences were considered significant at $p < 0.05$.

3 – RESULTS

3.1. Maternal behaviour

As shown in figure 1, maternal behaviour was greatly affected by the experimental treatments. There was indeed a significant difference for the duration of pup-retrieval ($H = 8.64$, $p = 0.013$) and the duration of pup-licking ($H = 8.18$, $p = 0.017$), whereas the presence in the nest was comparable among the experimental groups ($H = 0.56$, $p = 0.756$). At PND 9, the mothers of the litters exposed to olfactory deprivation spent less time to bring the young back to the nest compared to untreated ($p = 0.009$) and sham group mothers ($p = 0.016$). In addition, the duration of pup-licking was increased in the litters exposed to olfactory deprivation compared to untreated ($p = 0.009$) and sham groups ($p = 0.917$ for pup-retrieval; $p = 0.602$ for pup- licking).

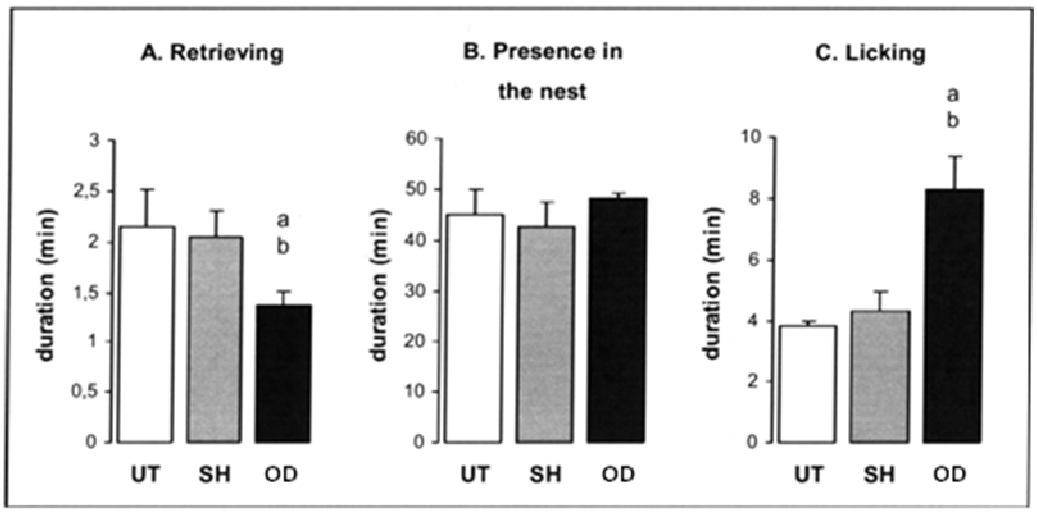

Figure 1. Maternal behaviour at 9 days of age in untreated group (UT), sham group (SH) and animals with olfactory deprivation at 8 days (OD).(A) Duration of pup-retrieval, (B) duration of presence in the nest and (C) duration of pup-licking. Values are means ± SE. $n = 5$ mothers per group. Analysis of Kruskal-Wallis, pup-retrieval: $H = 8.64$, $p = 0.013$; presence in the nest: $H = 0.56$, $p = 0.756$; pup-licking: $H = 8.18$, $p = 0.017$. Analysis of Mann-Whitney U-test: $^{a}p < 0.05$ versus untreated; $^{b}p < 0.05$ versus sham.

3.2. Suckling behaviour

Figure 2 shows that the suckling behaviour was affected by the experimental treatments.

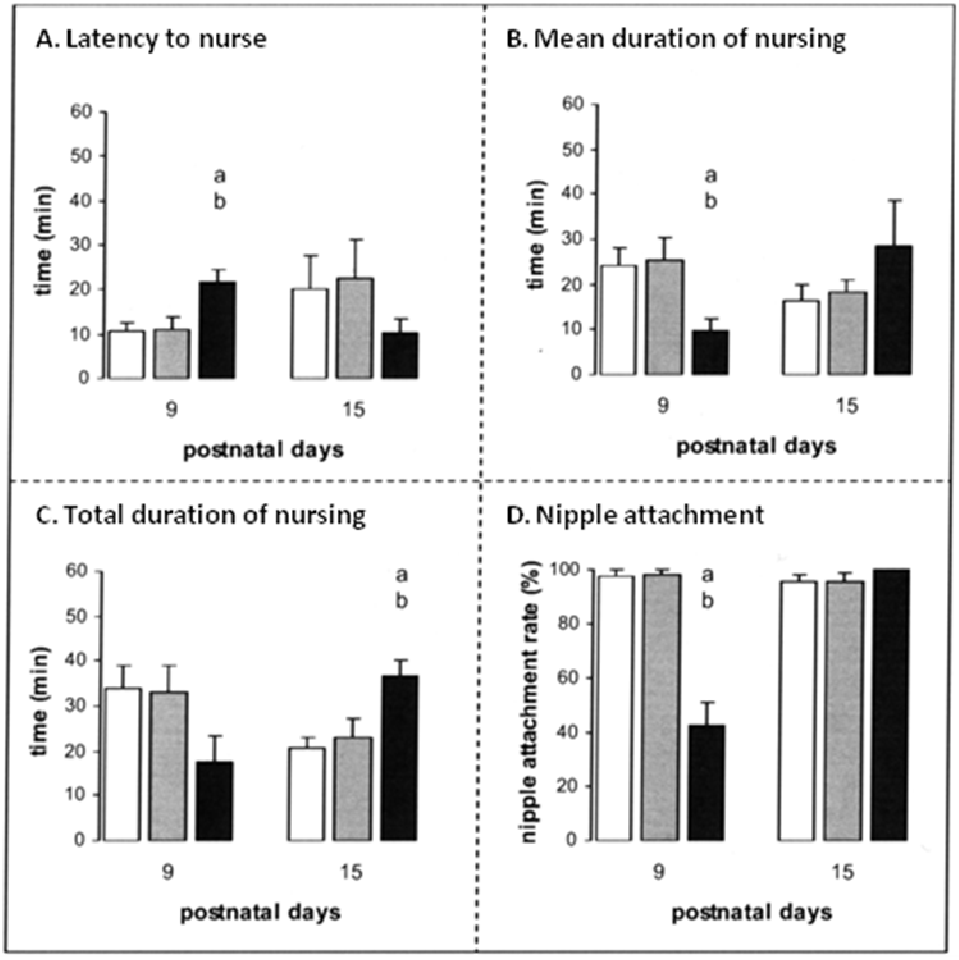

Figure 2. Suckling behaviour at 9 and 15 days of age in untreated group (□), sham group ( ) and animals with olfactory deprivation at 8 days (■) (A) Latency of the first alimentary event, (B) mean duration of alimentary events, (C) total duration of alimentary events and (D) maximum rate of nipple attachment. Values are means ± SE. n = 5 mothers per group; 6 < n < 9 alimentary events per age and per group. Analysis of Kruskal-Wallis, latency, PND 9: H = 6.62, p = 0.037; PND 15: H = 0.56, p = 0.756. Mean duration, PND 9: H = 10.09, p = 0.006; PND 15: H = 5.43, p = 0.066. Total duration, PND 9: H = 4.38, p = 0.112; PND 15: H = 6.66, p = 0.036. Nipple attachment, PND 9: H = 14.73, p = 0.0006; PND 15: H = 2.21, p = 0.332. Analysis of Mann-Whitney U-test: $^{a}p < 0.05$ versus untreated; $^{b}p < 0.05$ versus sham.

At PND 9, there was indeed a significant difference for the latency to nurse (H = 6.62, p= 0.037), the mean duration of nursing (H = 10.09, p = 0.006) and the rate of nipple attachment (H = 14.73, p = 0.0006). Latency to nurse was indeed higher in the pups exposed to olfactory deprivation compared to untreated and sham operated pups (p = 0.028 in both cases). On the other hand, the mean duration of nursing and the rate of nipple attachment were reduced in the litters exposed to olfactory deprivation compared

to untreated (p = 0.007 for the mean duration; p = 0.002 for the nipple attachment) and sham pups (p = 0.01 for the mean duration; p = 0.002 for the nipple attachment). These differences disappeared at PND 15 (H = 0.56, p = 0.756 for the latency; H = 5.43, p =0.066 for the mean duration; H = 2.21, p = 0.332 for the nipple attachment); at this age the total duration of nursing was affected by the experimental treatments (H = 6.66, p = 0.036). Indeed the total duration of nursing was higher at PND15 in the pups exposed to olfactory deprivation compared to untreated (p = 0.016) and sham pups (p = 0.047). For all the studied ages and parameters, there were no significant differences between untreated and sham groups ($0.602 < p < 0.999$).

3.3. Return to the nest

When tested for their reactions in the two-choice situation "nest sawdust versus clean sawdust" (figure 3 and 4), a significant difference was measured at PND 9 for the percentage of pups carrying out a choice ($3.88 < \chi^2 < 10.41$, $0.001 < p < 0.048$), for the return rate to the nest ($4.47 < \chi^2 < 5.32$, $0.021 < p < 0.034$), for the latency of the first choice [F (2, 66) = 14.92; $p < 0.0001$] and for the time spent in each arm of the T-maze [F (2, 66) = 23.84; $p < 0.0001$ for nest sawdust; F (2, 66) = 7.93; p = 0.0008 for center arm F (2, 66) = 10.00; p = 0.0002 for clean sawdust]. Some of these differences were reduced at PND 15. At this age, there was indeed no more significant difference for the percentage of rats carrying out a choice ($0.002 < \chi^2 < 1.60$, $0.206 < p < 0.965$) and the latency of the first choice [F (2, 66) = 2.03; p = 0.140]; whereas the return rate to the nest ($0.31 < \chi^2 < 5.56$, $0.018 < p < 0.580$) and the time spent in the nest side [F (2, 66) = 4.48; p = 0.001] remained significantly different.

At PND 9, pups exposed to olfactory deprivation carried out less choice than untreated (χ^2 = 3.88, p = 0.048 in females; χ^2 = 5.96, p = 0.015 in males) and sham pups (χ^2 = 5.17, p = 0.023 in females; χ^2 = 10.41, p = 0.001 in males). Furthermore, olfactory deprived pups exhibited a decreased rate of return to the nest in both females (χ^2 = 5.32, p = 0.021 versus untreated and sham) and males (χ^2 = 4.47, p = 0.034 versus untreated; χ^2 = 4.85, p = 0.028 versus sham). These pups showed also a greater latency of the first choice compared to untreated (p = 0.0002 in females; p = 0.006 in males) and sham pups (p = 0.0006 in females; p = 0.001 in males). Pups exposed to olfactory deprivation spent less time on the nest side ($p < 0.0001$ in all cases) to the advantage of the clean sawdust side (females: p = 0.003 versus untreated and sham; males: p = 0.010 versus

untreated, $p = 0.009$ versus sham) and of the center arm side (females: $p = 0.046$ versus untreated, $p = 0.023$ versus sham; males: $p = 0.008$ versus untreated, $p = 0.003$ versus sham). Olfactory deprived pups presented a time distribution close to random distribution (about 33 % of time in each arm of the maze).

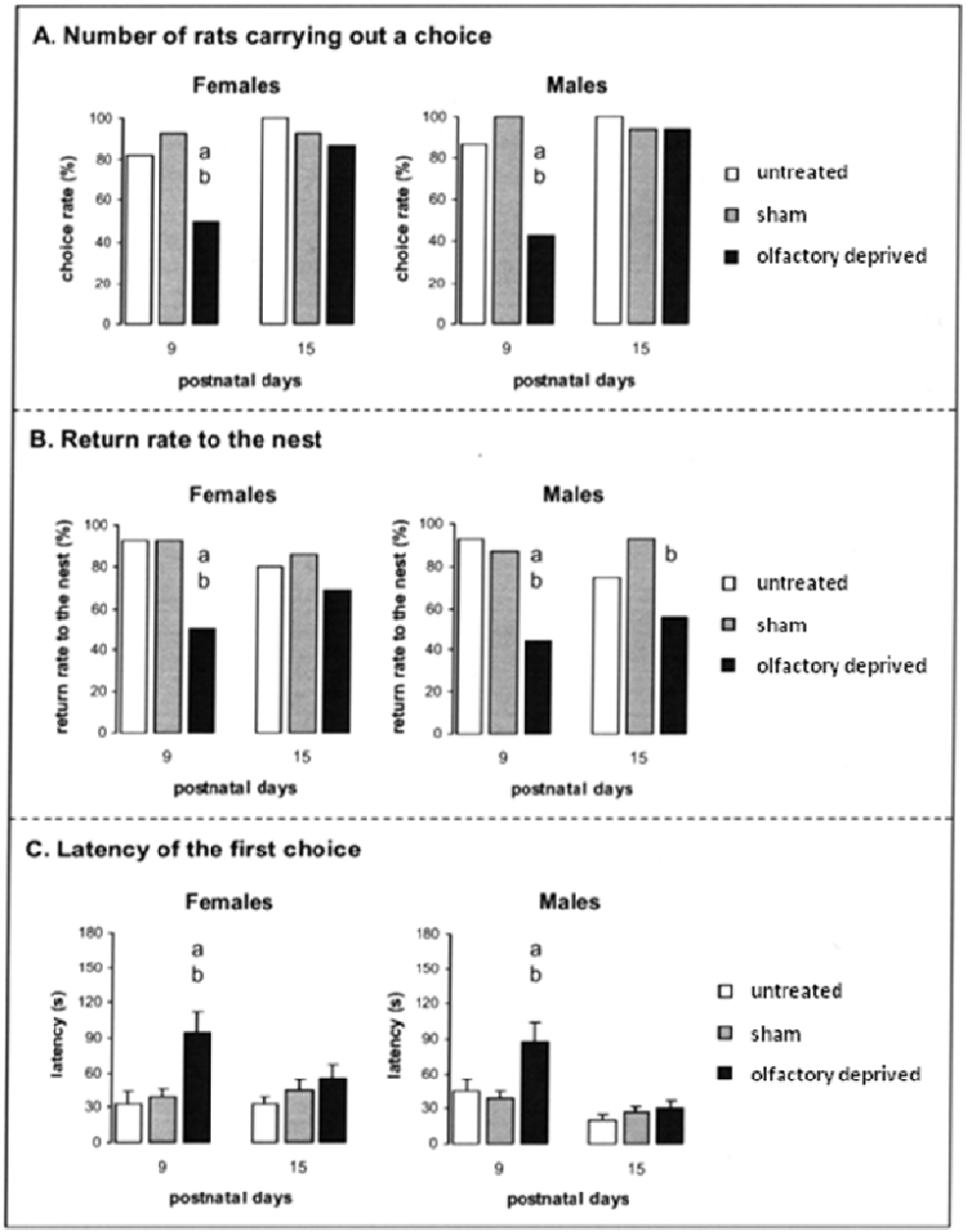

Figure 3. Impact of early nasal obstruction on animals' behaviour in a two-choice situation: "nest sawdust vs. clean sawdust". (A) Number of rats carrying out a choice, (B) rate of return to the nest in first intention and (C) latency of the first choice at 9 and 15 days of age in untreated group, sham group and animals with olfactory deprivation at 8 days. Values are percentage (A and B) or means ± SE (C). $n = 15$ rats per sex and per group. Latency of the first choice: analysis of two-way ANOVA, treatment effect: PND 9: $F = 7.56$ at two degrees of freedom, $p < 0.0001$; PND 15: $F = 1.93$ at two degrees of freedom, $p = 0.101$. Analysis of chi- square for independent data (A and B) or analysis of Fisher PLSD (C: $^{a}p < 0.05$ versus untreated; $^{b}p < 0.05$ versus sham.

At PND 15, males exposed to olfactory deprivation still showed a lower rate of return to the nest compared to sham males only ($\chi^2 = 1.25$, $p = 0.264$ versus untreated; $\chi^2 = 5.56$, $p = 0.018$ versus sham), whereas there was no more significant difference in females ($\chi^2 = 0.50$, $p = 0.481$ versus untreated; $\chi^2 = 0.31$, $p = 0.580$ versus sham). The time spent on the nest side remained inferior in olfactory deprived females compared to untreated ($p = 0.010$) and SH females ($p = 0.0004$) and in olfactory deprived males compared to sham males only ($p = 0.159$ versus untreated; $p = 0.003$ versus sham). Nevertheless, that did not result any more in significant differences on the time spent in the clean sawdust side [F (2, 66) = 2.32; $p = 0.051$] and in the center arm [F (2, 66) = 2.11; $p = 0.073$]. At last, for all the studied parameters, there never was significant difference between untreated and sham pups ($0.099 < p < 0.999$).

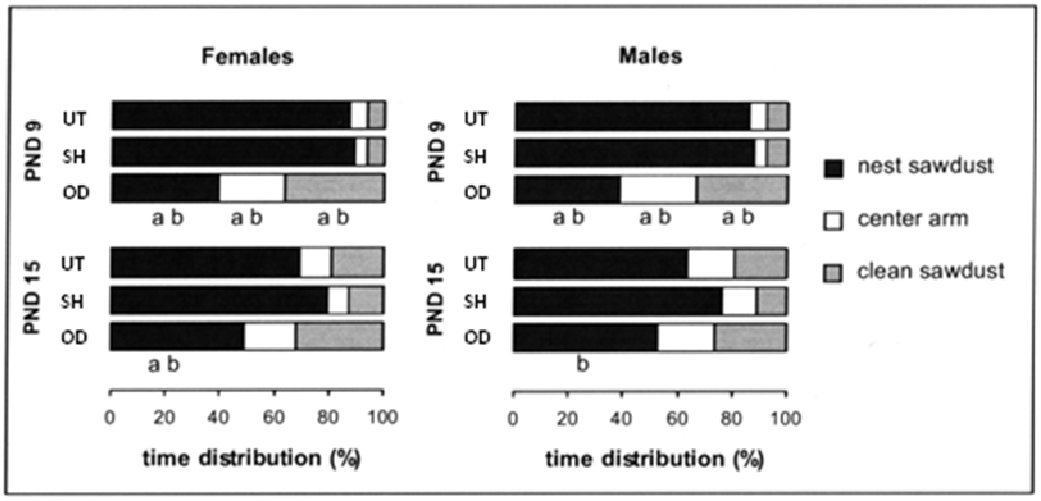

Figure 4. Distribution of the time spent in each arm of the T-maze in a two-choice situation, "nest sawdust vs. clean sawdust", at 9 and 15 days of age in untreated group (UT), sham group (SH) and animals with olfactory deprivation at 8 days (OD). Values are means. $n = 15$ rats per sex and per group. PND, postnatal days. Analysis of two-way ANOVA, treatment effect: PND 9: $F = 9.58$ at two degrees of freedom, $p < 0.0001$ for nest sawdust; $F = 3.27$ at two degrees of freedom, $p = 0.011$ for center arm; $F = 4.02$ at two degrees of freedom, $p = 0.003$ for clean sawdust. PND 15: $F = 4.48$ at two degrees of freedom, $p = 0.001$ for nest sawdust. Analysis of Fisher PLSD: $^a p < 0.05$ versus untreated; $^b p < 0.05$ versus sham.

3.4. Growth parameters

As shown in table 1, there was no significant differences in body weight at PND 9 between the experimental groups [F (2, 71) = 0.64; $p = 0.532$]. In contrast, a significant difference was detected at PND 15 [(2, 71) = 21.76; $p < 0.0001$]. Pups exposed to olfactory deprivation had a lower body weight in both females ($p = 0.0004$ *versus*

untreated, $p = 0.002$ *versus* sham) and males ($p < 0.0001$ *versus* untreated and sham). Compared to untreated group, the reduction was 20 % in females and 24 % in males. The experimental treatments also affected the ponderal gain over the period PND 9 - PND 15 [F (2, 71) = 23.29; $p < 0.0001$], which was reduced under olfactory deprivation in both females ($p < 0.0001$ *versus* untreated, $p = 0.0004$ *versus* sham) and males ($p = 0.0001$ *versus* untreated, $p = 0.0002$ *versus* sham). Compared to the untreated group, the reduction was 52 % in females and 61 % in males. Besides, there was no significant difference between untreated and sham animals ($p = 0.597$ in females, $p = 0.921$ in males).

		Weight (g)			Length (mm)		
		PND 9	PND 15	gain	PND 9	PND 15	gain
Females	UT	16.7 ± 0.6	29.8 ± 1.5	13.1 ± 1.5	72.2 ± 0.6	85.8 ± 1.3	13.6 ± 1.4
	SH	16.9 ± 0.5	29.1 ± 1.5	12.2 ± 1.6	70.0 ± 0.9	84.1 ± 0.9	14.1 ± 1.5
	OD	17.7 ± 0.5	24.0 ± 0.9[a, b]	6.3 ± 1.1[a, b]	69.0 ± 0.6[a]	77.8 ± 1.2[a, b]	8.8 ± 1.3[a, b]
Males	UT	18.6 ± 0.6	30.3 ± 1.0	11.8 ± 0.7	71.3 ± 0.6	85.8 ± 0.5	14.5 ± 0.9
	SH	18.4 ± 0.4	30.0 ± 0.8	11.6 ± 0.8	71.5 ± 0.8	86.2 ± 0.6	14.8 ± 0.8
	OD	18.5 ± 0.4	23.1 ± 1.2[a, b]	4.6 ± 1.1[a, b]	68.3 ± 1.1[a, b]	77.3 ± 1.0[a, b]	9.1 ± 1.7[a, b]

Table 1. Body weight (g) and body length (mm) at 9 and 15 days of age in untreated group (UT), sham group (SH) and animals exposed to olfactory deprivation at 8 days (OD). The ponderal and longitudinal gains of each individual ($n = 15$ rats per sex and per group) were calculated over the period PND 9 - PND 15. Values are means ± SE. PND, postnatal day. Analysis of two-way ANOVA, treatment effect: body weight at PND 15 and ponderal gain: $F = 8.80$ at two degrees of freedom, $p < 0.0001$; body length at PND 9: $F = 3.25$ at two degrees of freedom, $p = 0.011$; body length at PND 15: $F = 19.12$ at two degrees of freedom, $p < 0.0001$; longitudinal gain: $F = 4.48$ at two degrees of freedom, $p = 0.001$. Analysis of Fisher PLSD: $^{a}p < 0.05$ versus untreated; $^{b}p < 0.05$ versus sham.

Table 1 shows also that the experimental groups exhibited different body lengths at both PND 9 [F (2, 71) = 7.10; $p = 0.002$] and PND 15 [F (2, 71) = 46.55; $p < 0.0001$]. Females exposed to olfactory deprivation presented indeed a lower body length at PND

9 compared to untreated females only (p = 0.012 *versus* untreated; p = 0.375 *versus* sham). In males, the nine-day old olfactory deprived animals showed a reduced body length compared to untreated and sham animals (p = 0.017 *versus* untreated; p = 0.007 *versus* sham). These differences tended to be accentuated at PND 15 in both females and males ($p < 0.0001$ *versus* untreated and sham in all cases). At this age, the body length was reduced by 9 % in females and by 10 % in males compared to untreated animals. The experimental treatments affected also the longitudinal gain over the period PND 9 - PND 15 [F (2, 71) = 10.94; $p < 0.0001$]. Indeed the longitudinal gain was reduced under olfactory deprivation in both females (p = 0.021 *versus* untreated, p = 0.005 *versus* sham) and males (p = 0.009 *versus* untreated, p = 0.003 *versus* sham). Compared to the untreated group, the reduction was 35 % in females and 37 % in males. There was no significant difference between untreated and sham animals (p = 0.808 in females, p = 0.897 in males).

3.5. Gastric content

As shown in figure 5, the specific weight of gastric content was affected by the experimental treatments at PND 9 [F (2, 42) = 16.17; $p < 0.0001$] and PND 15 [F (2, 42) = 6.86; p = 0.003]. At PND9, animals exposed to olfactory deprivation exhibited a decrease of gastric content weight in both females (p = 0.0005 *versus* untreated; p = 0.004 *versus* sham) and males ($p < 0.0001$ *versus* untreated; p = 0.0002 *versus* sham).

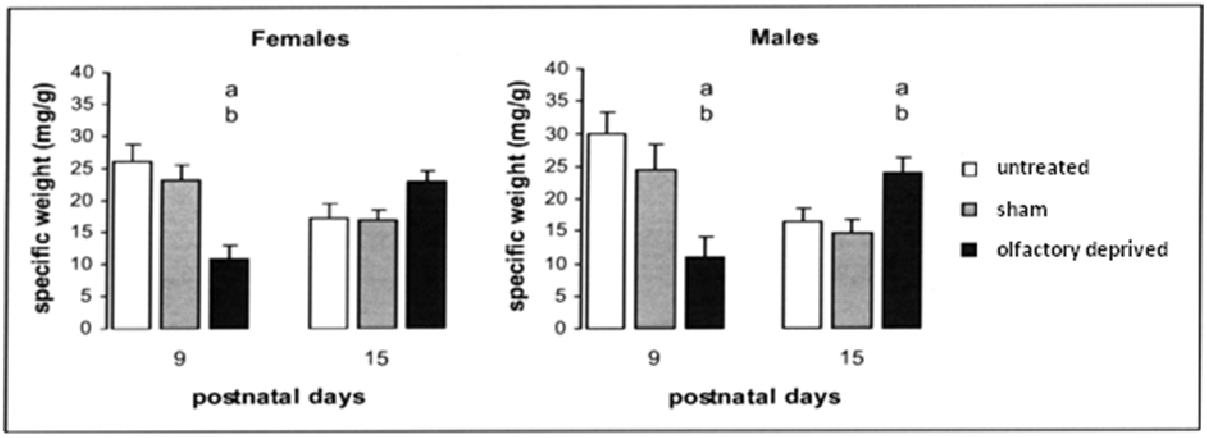

Figure 5. Specific weight of gastric content at 9 and 15 days of age in untreated group, sham group and animals with olfactory deprivation at 8 days. Values are means ± SE. n = 8 rats per sex, per age and per group. Analysis of two-way ANOVA, treatment effect: PND 9: F = 7.30 at two degrees of freedom, $p < 0.0001$; PND 15: F = 3.18 at two degrees of freedom, p = 0.016. Analysis of Fisher PLSD: $^{a}p < 0.05$ versus untreated; $^{b}p < 0.05$ versus sham.

Compared to untreated animals, the reduction was 59 % in females (10.8 ± 2.2

mg/g versus 26.0 ± 2.7 mg/g) and 64 % in males (10.9 ± 3.1 mg/g versus 29.9 ± 3.3 mg/g). At PND 15, this difference tended to be reversed. Indeed specific weight of gastric content was significantly higher at PND 15 in males exposed to olfactory deprivation (+ 31% and $p = 0.026$ *versus* untreated; +0.003 *versus* sham), although no significant difference was detected in females ($p = 0.074$ *versus* 38% and $p =$ untreated; $p = 0.061$ *versus* sham). Finally, whatever the age and the sex considered, there was no significant difference between untreated and sham animals ($0.226 < p < 0.930$).

3.6. Corticosterone assay

As shown in figure 6, plasma corticosterone levels were significantly different between the experimental groups at PND 9 [F (2, 42) = 21.40; $p < 0.0001$] and PND 15 [F (2, 42) = 23.60; $p < 0.0001$]. 24 hr after the treatment, olfactory deprivation was associated with an augmentation of corticosterone concentration in both sexes (females: $p = 0.0003$ *versus* untreated and sham; males: $p = 0.0001$ *versus* untreated, $p = 0.0004$ *versus* sham).

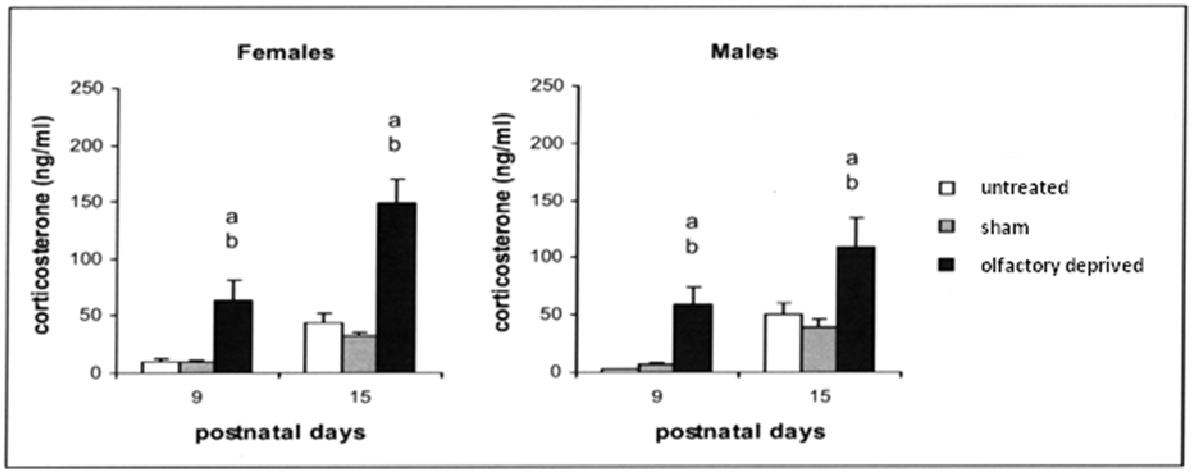

Figure 6. Plasma corticosterone levels at 9 and 15 days of age in untreated group, sham group and animals with olfactory deprivation at 8 days. Values are means ± SE. $n = 8$ rats per sex, per age and per group. Analysis of two-way ANOVA, treatment effect: PND 9: $F = 8.63$ at two degrees of freedom, $p < 0.0001$; PND 15: $F = 10.30$ at two degrees of freedom, $p < 0.0001$. Analysis of Fisher PLSD: $^{a}p < 0.05$ versus untreated; $^{b}p < 0.05$ versus sham.

At PND 15, plasma corticosterone level remained significantly higher in olfactory deprived females (149.5 ± 20.8 ng/ml) compared to untreated (44.2 ± 8.1 ng/ml, $p < 0.0001$) and sham females (32.3 ± 2.9 ng/ml, $p < 0.0001$). Plasma corticosterone levels were significantly increased also in olfactory deprived males (109.0 ± 24.6 ng/ml)

compared to untreated males (51.0 ± 8.7 ng/ml, $p = 0.01$) and sham males (39.5 ± 6.3 ng/ml, $p = 0.003$). The values of plasma corticosterone were comparable between untreated and sham animals in both sexes at the two ages (PND9: $p = 0.989$ in females, $p = 0.742$ in males; PND15: $p = 0.683$ in females; $p = 0.617$ in males).

3.7. Thyroid hormone assays

Figure 7A shows that plasma thyroxin levels were significantly different between the experimental groups at PND9 [F (2, 42) = 36.36; $p < 0.0001$] and PND 15 [F (2, 42) = 13.94; $p < 0.0001$] and between the sexes at PND9 only [F (1, 42) = 6.21; $p = 0.017$]. At PND 9, 24h after the treatment, thyroxin concentration was significantly reduced in sham females compared to untreated females ($p = 0.01$). The same tendency was observed in sham males although the difference compared to untreated males was not significant ($p = 0.06$).

Moreover, females exposed to olfactory deprivation exhibited a significant decrease of thyroxin levels compared to untreated and sham females, but also compared to their male counterparts ($p < 0.0001$ in all cases). In nine-day old olfactory deprived males, a significant decrease of thyroxin concentration was detected compared to untreated males only ($p = 0.009$ *versus* untreated; $p = 0.43$ *versus* sham). At PND 15, there was no more significant difference between untreated and sham animals ($p = 0.07$ in females; $p = 0.34$ in males). In contrast, animals exposed to olfactory deprivation showed a significant decrease of plasma thyroxin level in both females ($p = 0.0002$ versus untreated; $p = 0.03$ versus sham) and males ($p = 0.008$ *versus* untreated; $p = 0.0005$ *versus* sham).

As shown in figure 7B, plasma triiodothyronin levels were significantly different between the experimental groups at PND 9 [F (2, 42) = 24.26; $p < 0.0001$] and PND 15 [F (2, 42) = 5.62; $p = 0.007$]. Contrary to what was observed for thyroxin, the triiodothyronin concentration was significantly increased at PND 9 in sham animals compared to untreated animals ($p = 0.007$ in females; $p = 0.001$ in males). At this age, individuals exposed to olfactory deprivation exhibited a significant diminution of triiodothyronin level compared to sham animals ($p = 0.0003$ in females; $p < 0.0001$ in males), whereas there was no significant difference compared to untreated animals ($p = 0.26$ in females; $p = 0.06$ in males). At PND 15, the triiodothyronin concentration remained lower in females exposed to olfactory deprivation compared to sham females

only (p = 0.19 versus untreated; p = 0.003 versus sham). There was no more significant difference between the males (0.14 < p < 0.70).

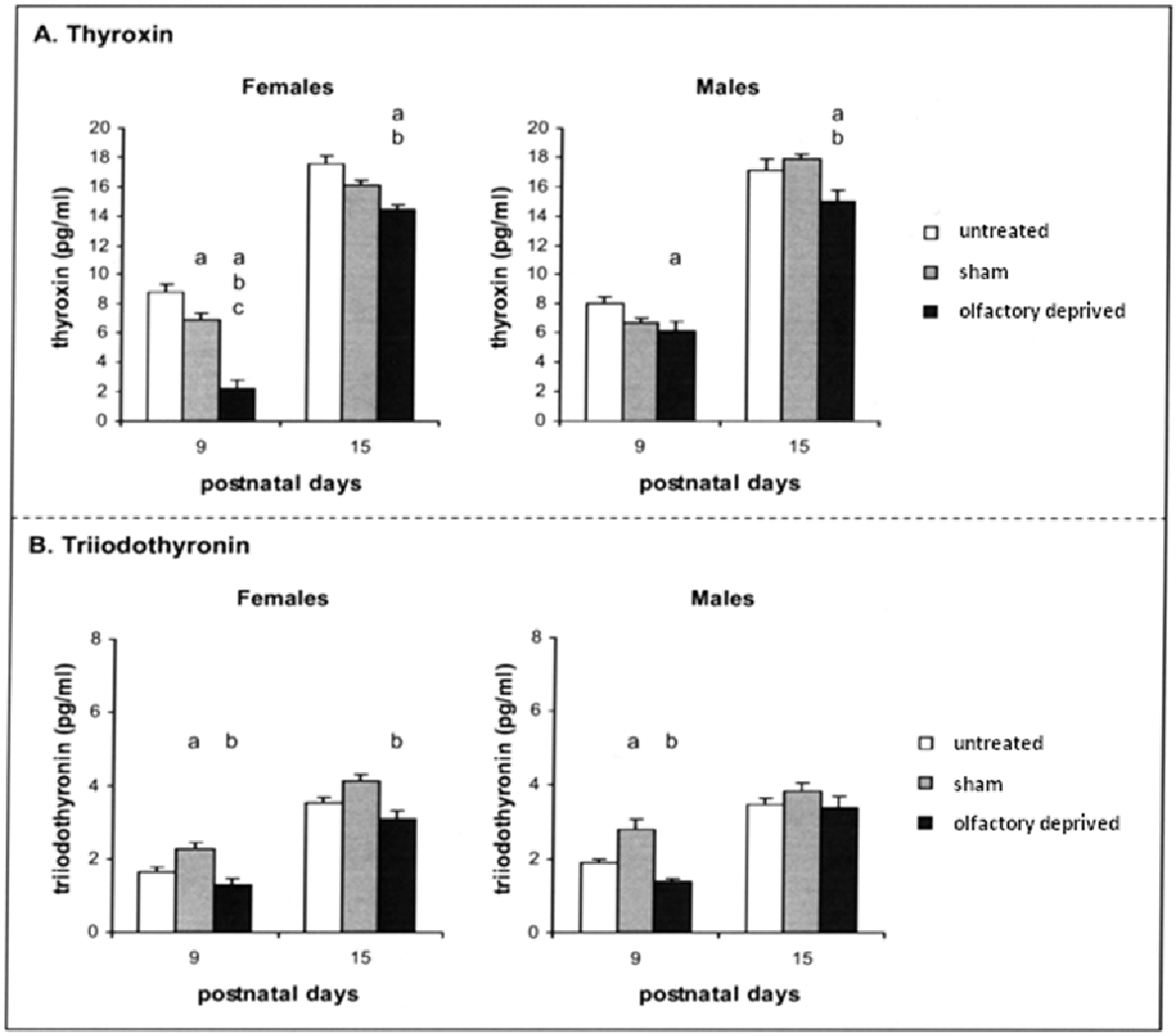

Figure 7. Plasma thyroxin (A) and triiodothyronin (B) levels at 9 and 15 days of age in untreated group, sham group and animals with olfactory deprivation at 8 days. Values are means ± SE. n = 8 rats per sex, per age and per group. Analysis of two-way ANOVA, treatment effect: PND 9: F = 20.27 at two degrees of freedom, p < 0.0001; PND 15: F = 6.53 at two degrees of freedom, p = 0.0002. Analysis of Fisher PLSD: ^{a}p< 0.05 versus untreated; ^{b}p < 0.05 versus sham.

4 - Discussion

Our results revealed that the mothers of olfactory deprived pups exhibited a decreased duration of pup-retrieval and an increased duration of pup-licking. Since early olfactory deprivation was associated with an increase of circulating corticosterone at both PND 9 and PND 15, we hypothesized that the mothers of olfactory deprived pups probably perceived the distress of their young via acoustic and/or chemical signals. Indeed it is well known that in distress situations, rodents emit vocalizations and

chemical signals which can be selectively recognized by recipients (Hofer & Shair, 1992; Ma et al., 1998). A higher level of vocalization in olfactory deprived animals could accelerate the retrieval behaviour and thus reduce the duration of pup-retrieval as observed in the olfactory deprived pups. Our results showed that the duration of pup-licking was greater in the olfactory deprived pups and this could also be related to the calls coming from the offspring. Indeed the isolation calls contribute to an increase in anogenital licking by the mother following retrieval (Brouette-Lahlou et al., 1992). The majority of total licking time is spent licking the pup's anogenital region, behaviour that stimulates reflexive defecation and urination (Moore, 1982). In addition to the influence of acoustic signals, anogenital licking is also strongly related to a pheromone present in the urine of the offspring (Brouette-Lahlou et al., 1999). We can thus suppose that the mothers of olfactory deprived pups could perceive the distress of their young via for example adrenal- mediated urinary metabolites, and consequently increased the licking duration (Pruett et al., 2008). Nevertheless, further investigations are necessary to clarify the nature of the sensory signals with which the mother perceived the distress of her young. Dehydration would be one signal to investigate as this would procedure a decrease in urine production thus encouraging the mothers to lick more frequently to try and stimulate urination.

Our results also revealed that suckling behaviour was affected by olfactory deprivation. At PND 9, the olfactory deprived pups exhibited indeed a greater latency to nurse, a lower mean duration of nursing and a decrease in nipple attachment rate. These modifications resulted in a diminution of the ingested milk quantity and growth retardation. Food intake was however restored, even reversed, with the reopening of the nostrils. Olfactory deprived animals presented then an increased of total duration of nursing and a higher quantity of ingested milk. It is well documented that olfaction plays a central part in the expression of suckling behaviour and notably in the young rat, in which the orientation to the nipples is pheromone-dependent (Blass & Teicher, 1980). On the other hand, it was shown that establishment of the nursing posture and milk ejections were dependent on the combined suckling stimuli of several pups (Stern & Johnson, 1990). Since the mothers of olfactory deprived pups were present at the nest and correctly cared for their pups, we conclude that the decreased food intake was related to the olfactory deprivation and to the associated difficulties to find the nipples. One clear possibility to further test this hypothesis would be to compare food intake in litters that exclusively comprise olfactory deprived pups with mixed litters

simultaneously comprising untreated, sham and olfactory deprived pups. Besides, duration of nursing and olfactory deprivation involves competition between the respiratory and alimentary processes (Kalogjera et al., 1991). Respiratory disturbance could thus represent a factor worsening the impact of olfactory deprivation on food intake. The assumption that the decrease of food intake was, at least partially, related to difficulties to find the nipples is also supported by the results concerning olfactory abilities. Once nasal breathing was restored pups were able to suckle for longer to "catch up" the lost growth.

Our results showed indeed that bilateral naris occlusion had a functional impact on olfactory ability that disturbed orientation to the nest 24 hr after treatment. The nine-day old olfactory deprived animals exhibited a lower rate of return to the nest and randomly distributed their time in the three arms of the maze. Olfactory deprived animals performed also less choice and presented an elevated latency of the first choice suggesting a perturbation of exploratory behaviour. These impairments could be explained by both a lack of olfaction and, as shown in our previous investigation (Gelhaye et al., 2006b), by an increased level of anxiety. Olfactory deprivation and exacerbated anxiety could indeed act together on exploratory behaviour. Besides, the differences observed for the number of choices and the latency of the first choice were totally disappeared at PND 15, whereas the return rate to the nest and the time spent in the nest side remained significantly different. These data suggest a fast recovery to a basal level of exploratory behaviour and a slower functional recovery of olfactory ability despite nostrils reopening. This could be explained by the fact that with the appearance of hearing and vision (approximately at PND 10 and PND 14, respectively), the relative role of olfaction in the expression of exploratory behaviour decreases gradually. On the other hand, naris occlusion produces an atrophy of the olfactory system's components demonstrated for both the olfactory bulb and the olfactory mucosa (Brunjes, 1994 ; Stahl et al., 1990). In particular, it was shown that after the induction of a naris occlusion, the return to normal breathing induced a restoration of the olfactory mucosa depth within a few days (Mirich & Brunjes, 2001). This recovery time could explain the delay in the functional recovery of the olfactory ability.

In order to know the impact of nutritional disturbance on the thyroid axis, T3 and T4 levels were assayed. Nutritional depletion is indeed known to impair the maintenance of hormonal homeostasis during the postnatal period (Oberkotter, 1988 ; Schmidt et al., 2002). First, nine-day old sham individuals presented an increase in T3

levels to the detriment of T4. Hence, we conclude that the maternal separation linked to the procedure, the anaesthesia by hypothermia and the burn administered on sham rats impaired thyroid function. This effect was however time-limited since no difference was detected at PND 15. The opposite impact on T3 and T4 concentrations suggested a modification in the peripheral metabolism of the thyroid hormones. T3 is the most active thyroid hormone from a metabolic point of view (Köhrle, 1996). It can be synthesized by the thyroid gland but is generated mainly in the peripheral tissues by conversion from T4 (Kelly, 2000). Different factors are known to exacerbate the peripheral conversion of T4 among which is cold exposure. This involves indeed an activation of the sympathetic nervous system which, through an increase in catecholamine secretion, accelerates the intracellular conversion from T4 (Silva & Larsen, 1983 ; Diano et al., 1998). The procedure accompanying olfactory deprivation could so increase peripheral conversion of T4. Second, olfactory deprived animals exhibited a reduction of T4 levels at both PND 9 and PND 15. T3 concentrations were only different compared to sham animals suggesting an additional mechanism with modification of peripheral metabolism. It was shown that nutritional deprivation had a suppressive impact on T4 and T3 concentrations (Diano et al., 1998 ; Kasdallah et al., 2005). However, our results failed to show a significant reduction in T3 levels in animals exposed to olfactory deprivation. This could be explained by a masking effect of the procedure accompanying olfactory deprivation. Olfactory deprivation itself and the associated procedure would thus have synergistic effects on T4 levels and antagonist effects on T3 levels. Lastly, the present investigation showed that the decrease in T4 concentrations was more pronounced in females exposed to olfactory deprivation. In basal or stressful conditions, levels of T4 are generally higher in adult males due to the presence of testosterone (Christianson et al., 1981 ; Kobal et al., 2000 ; Waner & Nyska 1988). It would be thus interesting to perform an exogenous administration of testosterone in order to clarify the mechanisms underlying the lower level of T4 observed in females exposed to early olfactory deprivation.

In conclusion, early olfactory deprivation disturbed mother-pup interactions and decreased offspring's food intake. These behavioural changes were associated with an increase in corticosterone levels and a decrease of T4 levels, which were more marked in females. This study suggested that the effects of early olfactory deprivation on peripheral physiological systems could be partly linked to a disturbance of mother-pup interactions and notably of food intake. The impairment of food intake via hormonal

modifications could thus explain the structural changes of orofacial muscles and the reduction of the lymphocyte proliferation observed under olfactory deprivation. Our previous study showed furthermore that the behavioural, endocrine and immunologic impacts of olfactory deprivation were more marked in females than in males at PND 21. Except for T4 levels, these sex-dependant changes are less obvious in the present study and could thus appear rather during the recovery time after spontaneous nostril reopening.

Article 2

Effects of short term forced oral breathing in rat pups on weight gain, hydration and stress

G. S. Padzys, S. N. Thornton, J. M. Martrette, M. Trabalon

***Physiology & Behavior*, 2011, 102-2, 175-180**

1 - Introduction

Nasal obstruction is considered a risk factor in sleep-discordered breathing (Rombaux *et al.,* 2005 ; Armengot *et al.,* 2008; Craig *et al.,* 2008) which has a very negative impact on quality of life in children and adults with increased daytime sleepiness (Udaka *et al.,* 2006 this symptom resembles that of obstructive sleep apnea (OSA) caused by episodes of upper airway obstruction leading to episodic hypercapnic hypoxia which alters upper airway muscle structure and fibre type expression (McGuire *et al.,* 2002). The most common clinical manifestations of OSA are nocturnal snoring, respiratory pauses, restless sleep and mouth breathing (Balbani *et al.,* 2005). This disturbed breathing is known to produce lethargy, cognitive impairment and sleep impairment, especially in children (Dempsey *et al.,* 2010 ; Jefferson, 2010).

Chronic nasal obstruction is a non-specific condition observed in many pathophysiological conditions e.g. allergic rhinitis, rhinosinusitis, adenoid hypertrophy and nasal polyps. Impaired nasal breathing results in obligatory oral breathing, which can be divided into two components: chronic absence of active nasal respiration that results in an olfactory deprivation (Meisami, 1976) and chronic mouth opening (Schlenker *et al,* 2000). Furthermore, in contrast to oral breathing, nasal breathing allows the optimal conditioning of inhaled air, clearing, moistening and warming the air before the gas exchange in the lungs (Betlejewski, 1998 ; Keck *et al.*, 2001).

Obligatory mouth breathing has been observed in human babies and has been associated with a number of conditions that could have both short and long term effects

on the physiology and thus behaviour of these infants later on in adolescence. Decreases in oxygen saturation and respiratory frequency, with an increase in arousal were observed with nasal occlusion in preterm infants (de Almeida *et al.*, 1994). If untreated oral breathing in children can induce long narrow faces, narrow mouths, high palatal vaults, dental malocclusion, gummy smiles and other effects like skeletal facial profiles. These children do not sleep well at night and this lack of sleep can adversely affect growth and academic performance (Subtelny, 1980; Jefferson, 2010).

Oral breathing has been associated with increased net water loss in healthy subjects (Svensson *et al.*, 2006). This would be expected given that oral breathing does not allow conditioning of the inhaled air or demoisturising of the exhaled air. However, in the case of suckling rat pups dehydration could be consequence of decreased feeding due to a difficulty imposed by oral breathing and suckling at the same time (Harding, 1986). This could depend also on the position of the pups in the hierarchy on the nipple, males before females and stronger pups before weaker ones (Blass & Teicher, 1980).

To our knowledge no work has been published on feeding and hydrational changes during total closure of the nostrils in young animals before any normal ageing processes could intervene. Thus, our hypothesis was that early total nasal obstruction (from postnatal day 9 to day 11) would be associated with a switch to forced oral breathing thus having a negative impact on feeding/suckling leading to decreases in body weight gain, stomach content and in overall body hydration. Furthermore, we wanted to investigate if these changes were maintained after spontaneous opening of the nostril as well as over the long term, i.e. up to adulthood (postnatal day 90).

2 - Materials and methods

2.1. Animal care

Male and female Wistar rats (origin IFFA- CREDO) were used in these experiments. These pups were born in the laboratory from twenty litters, culled to 7 pups per litter to ensure normal body growth. The animals were housed in standard cages under controlled temperature conditions (22 ± 1°C). Food (pellet of 12 mm, Harlan Interfauna Iberica SA) and water were available *ad libitum* throughout the

experiment. From birth, the rats were kept on a reversed 12:12 light-dark cycle (dark period 08:00-20:00h).

2.2. Nasal obstruction procedure

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (no. 85-23, revised 1996), the recommendations of the European Community Council for the Ethical Treatment of Animals (no. 86/609/EEC) and the regulations of the University of Nancy 1. All efforts were made to minimize animal suffering.

At 8 days of age (D8), the litters were first anesthetized by hypothermia (10 min at -18 ◦C). Animals were weighed and they were then semi randomly divided into one control group and one experimental group (oral breathing). Bilateral nasal obstruction resulting in forced oral breathing was performed in experimental animals (at least 7 per age and per sex) as described previously by Gelhaye *et al.* (2006a, 2006b). The selected method consisted in cauterizing the external nostrils, which is the most common and simple procedure allowing reversible nasal obstruction in neonates. The tissue surrounding the external nostrils was burned by placing a surgical cauterizing instrument (1 mm in diameter) on the nostrils, consequently occluding the orifice of the nostrils without mechanical or chemical damage to the olfactory mucosa. This procedure induced complete nasal obstruction between D8 and D11 with 100 % of the nostrils reopened at D15. The experiments were conducted during complete nasal obstruction (D9 and D11), during reopening of nasal (D13), one day (D15) and 90 days (D90) after post-reopening of the nostrils. As shown schematically in Figure 1.

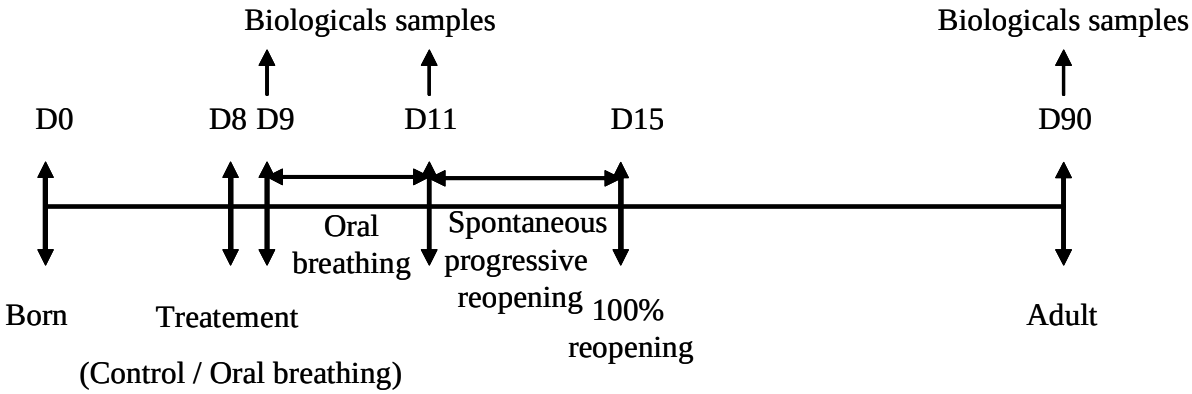

Figure 1. Time line of experimental protocol.

In the control group (at least 7 per age and per sex), the nostrils were not sealed but the cauterizing instrument was placed about 1-2 mm above each nostril. After cauterization, the nostrils were washed with chlortetracycline (Aureomycine Evans 3%) to prevent infection. The pups were warmed (37°C) for 30 mn and returned to their mothers.

2.3. Sample collection

In their home cages mothers and pups were videotaped on D9 and D11 during the dark phase between 09.00 hr and 12.00 hr. Duration of pup-licking, ie when the mother licked the body and/or the anogenital region of the pups, was analysed.

2.4. Sample collection

Seven male rats per group (control and oral breathing) per age and per sex (D9, D11, D13, D15 and D90), were randomly removed, immediately sacrificed, weighed and intracardiac blood samplings (500 – 1000 µl) were taken between 11h and 12 h for hormonal measurements. Blood was collected within 1-2 min into sterile heparinised syringes fitted with a 26-G needle. Plasma was immediately separated from cells by centrifugation at 4°C (15 min at 3000 rpm) then the extracts were aliquoted and stored at -18°C until the time of the assay.

Concentration of blood glucose was determined using colorimetric method after enzymatic oxidation in the presence of glucose oxidase (Glucose-test, Randox, UK). The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as indicator.

Osmolality was determined using a Roebling Automatic Osmometer with 100 µl of plasma. After blood sampling, the pocket of milk in the stomach was removed and weighed to evaluate the gastric contents.

2.5. Hormone assays

Vasopressin concentrations were measured without an extraction procedure, using a commercially available EIA kit and according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentration of vasopressin in plasma samples was calculated from a standard curve and expressed as pg/ml. The intra- and inter-assay coefficients of variation were, 10.2 % and 10.6 %.

Corticosterone concentrations were measured without an extraction procedure, using a commercially available EIA kit and performed according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentration of corticosterone in plasma samples was calculated from a standard curve and expressed as ng/ml. The intra- and inter-assay coefficients of variation were under 8.4 % and 13.1 %,

2.6. Statistical analysis

The results were expressed as group means ± SEM. Group differences were determined using analysis of variance (ANOVA). Analysis of Specific mean comparisons were then made using PLSD Fischer test. Differences were considered significant at $P < 0.05$.

3 - Results

3.1. Body weight

Before the treatment, at 8 days of age, the weights of control and oral breathing pups were not significantly different ($F_{3,109} = 4.50$, $P < 0.22$): males (17.8 ± 0.5 g and 17.9 ± 0.3 g) and females (17.6 ± 0.2 g and 17.2 ± 0.4 g).

Figure 2 shows that in males there was a significant difference ($F_{7,49} = 4.17$, $P < 0.002$) in body weight at D9 and D11 between control and oral breathing rats. No treatment differences were observed during the reopening of nostrils from D13 and after full opening by D15.

In females no difference was observed 24h after treatment. There was a significant decrease for body weight at D11 and D13 in the oral breathing group ($F_{7,49} = 5.50$, $P < 0.001$).

No differences were observed at D15 between control and oral breathing rats. The weights were similar at adulthood (394 – 408 g for males and 239 – 244 g for females).

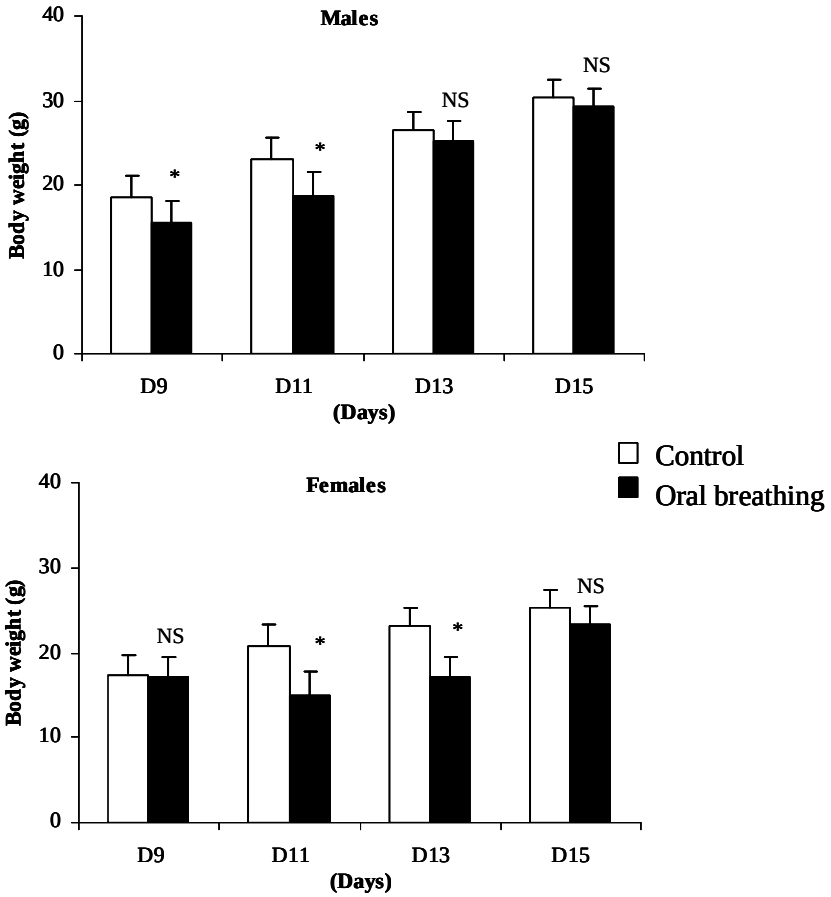

Figure 2. Effects of chronic oral breathing on body weight (g), at age 9, 11, 13, and 15 days in controls and animals exposed to nasal obstruction. Values are means ± S.E.M. (n = 7 / group / age). ANOVA summary: * significantly different from control group at $P < 0.001$; NS: no significantly different.

3.2. Gastric content

Gastric contents were removed and weighed and taken as an indication of food intake (Fig. 3). A significant reduction of gastric content weight was observed at D9 and D11 in rat pups in the oral breathing group compared to the control group ($F_{7,49} = 5.93$ and 4.94, $P < 0.0001$). No treatment differences were observed between the two groups around the time of the reopening of the nostrils (D13 and D 15).

These results were obscured somewhat by the fact that at the measurement time point some of the rat pups of all groups and sexes, but most particularly of the oral breathing group, did not have any gastric content and therefore, their stomach contents were not weighed. Of the 10 rats in the forced oral breathing group on both days 9 and

11, 4 of the males did not contain any content and in the females 6 were empty. In the controls for both days and both sexes 2 were empty.

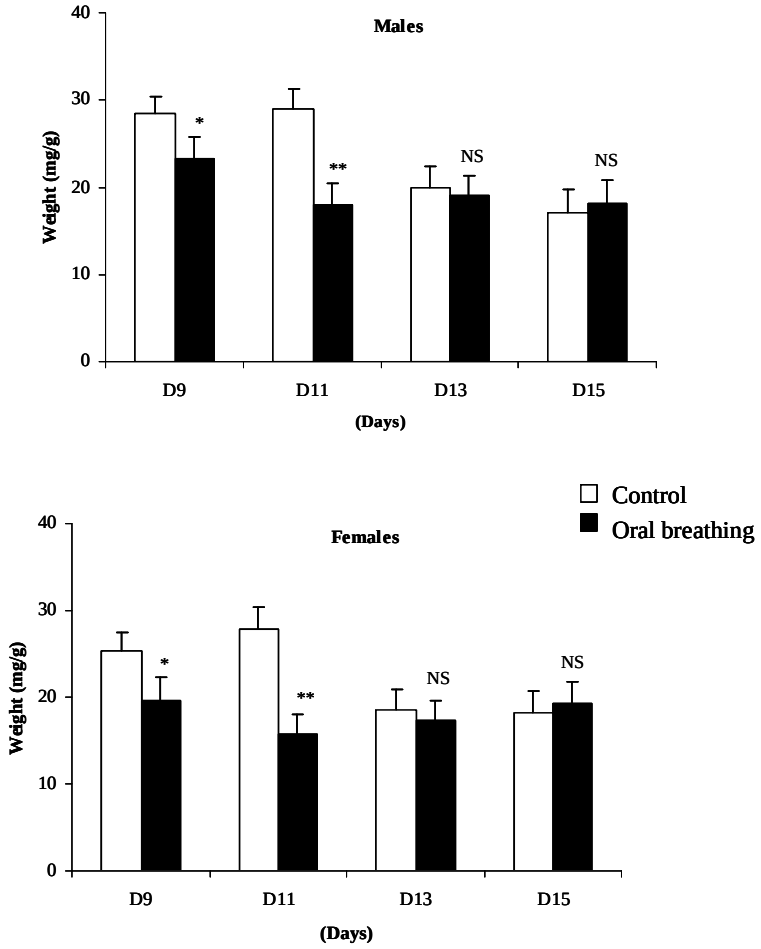

Figure 3. Effects of chronic oral breathing on content gastric (mg/g), at age 9, 11, 13, and 15 days in controls and animals exposed to nasal obstruction. Values are means ± S.E.M. (n = 7 / group / age). ANOVA summary: * significantly different from control group at $P < 0.01$; ** significantly different at $P < 0.001$; NS: no significantly different.

3.3. Glycemia

To determine if the oral breathing were associated with changed the glycemia, plasma glucose levels were obtained. No differences were observed in male plasma glucose levels between control and oral breathing rats: 145 ± 7 mg/dl *vs* 140 ± 7 mg/dl.

In females twenty-four hours after the treatment nasal obstruction was associated with a reduction of glycemia which was maintained until D11: 112 ± 3 mg/dl *vs* 135 ± 3 mg/dl ($F_{7,49} = 4.74$, $P < 0.02$).

No differences were observed after the reopening of nostrils between control and oral breathing rats at D15 and then at D90: (132 – 139 mg/dl for males and 131 – 147 mg/dl for females).

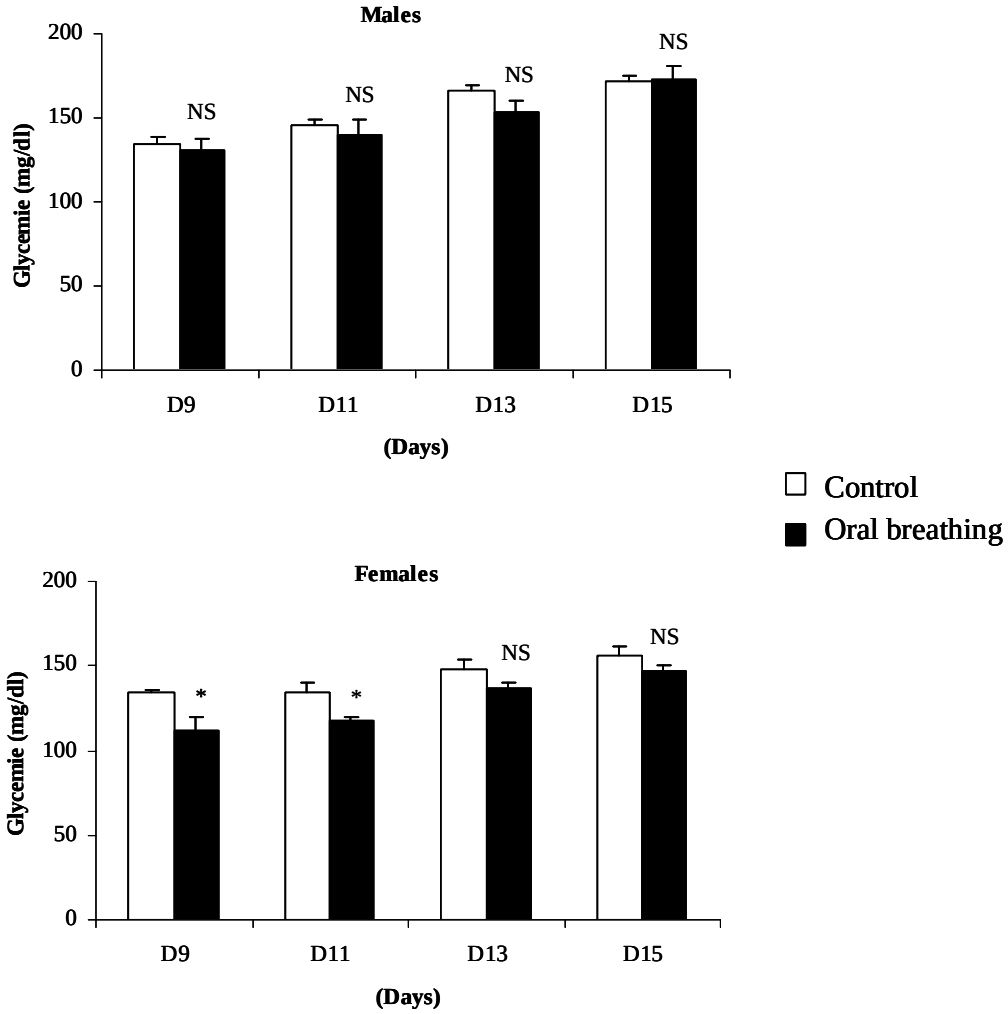

Figure 4. Impact of early nasal obstruction on glycemie levels at 9, 11, 13, and 15 days old in control and oral breathing animal. Values are means ± S.E.M (n = 7 rats/group/age). ANOVA summary: *significantly different from control group at $P < 0.05$; NS: no significantly different.

3.4. Osmolality, and vasopressin

To determine if chronic oral breathing was associated with dehydration, plasma osmolality and vasopressin concentrations was evaluated.

Figure 5 shows that in both male and female rat pups an increase in plasma osmolality was observed during the nasal obstruction period: 300 – 321 mosmol/kg H_2O compared with the controls (278 mosmol/kg H_2O; $F_{7,49}$ = 23.86 and 51.14, $P < 0.0001$). Upon reopening of the nostrils after D13 plasma osmolality returned to control values: 280 mosmol/kg H_2O.

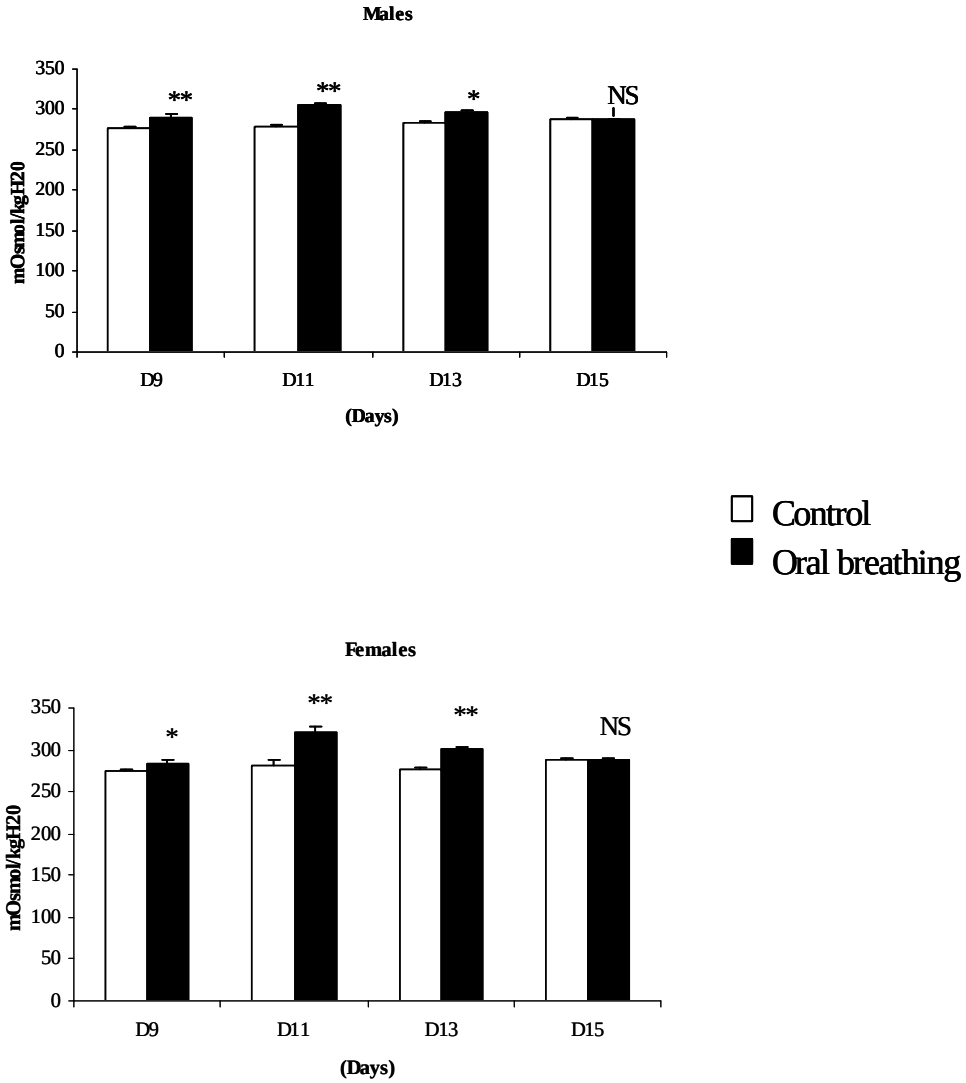

Figure 5. Impact of early nasal obstruction on plasma osmolarity levels at 9, 11, 13, and 15 days old in control and oral breathing animal. Values are means ± S.E.M (n = 7 rats/group/age). ANOVA summary: * significantly different from control group at $P < 0.005$; ** significantly different at $P < 0.0001$; NS: no significantly different.

Figure 6 shows the concentration of plasma vasopressin. Similar to the changes seen in plasma osmolality, the concentration of vasopressin in both male and female rat pups increased significantly ($F_{7,49}$ = 10.80, P < 0.0001) during the period of nasal obstruction: from 20 pg/ml until 60 pg/ml in females and from 19 pg/ml until 36 pg/ml in males.

No differences were observed between the control and the oral breathing groups in either male of female rat pups after reopening of the nostrils, even into adulthood: 22 pg/ml.

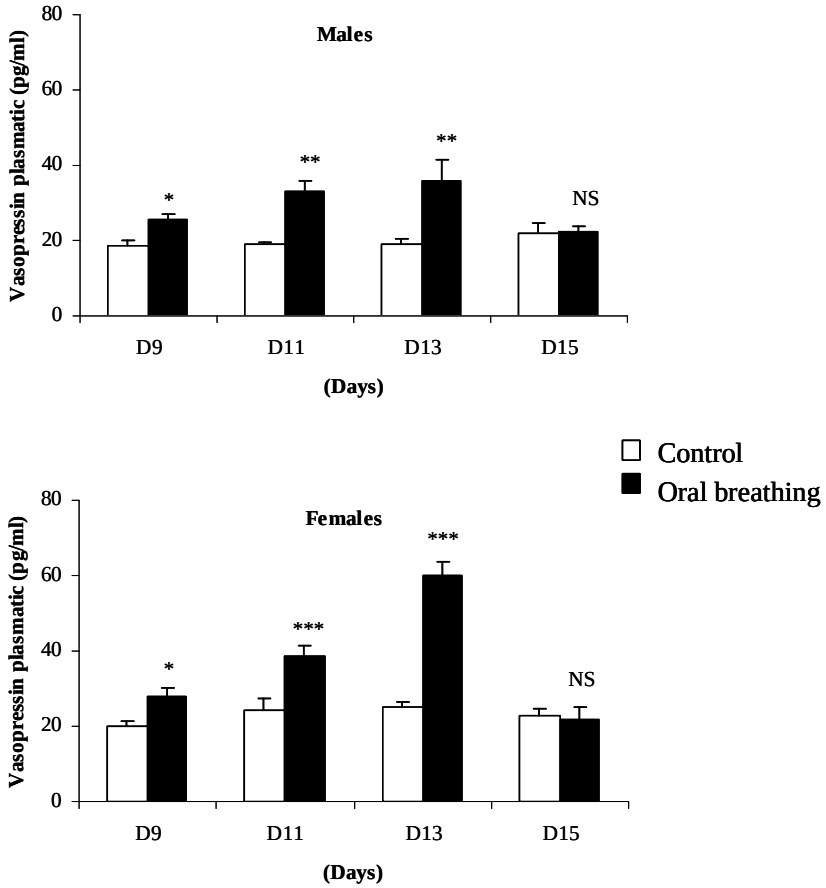

Figure 6. Impact of early nasal obstruction on plasma vasopressine level at 9, 11, 13, and 15 days old in control and oral breathing animal. Values are means ± S.E.M (n = 7 rats/group/age). ANOVA summary: * significantly different from control group at P < 0.01; ** significantly different at P < 0.001, *** significantly different at P < 0.0001, NS: no significantly different.

3.5. Licking rates

The only observations we obtained for this were on day 9 and 11 where licking was found to be greatly enhanced in mothers with nasally obstructed pups. Preliminary results: day 9, control mothers spent 281 ± 22 sec per pup versus 469 ± 54 sec per pup

for the mothers with obstructed nose pups ($F_{1,12}$ = 4.89, P < 0.03); day 11, 261 ± 28 sec for the controls and 554 ± 55 sec for the mothers of blocked nose pups ($F_{1,12}$ = 4.75, P < 0.04).

3.6. Corticosterone levels

Short term oral breathing produced a significant increase ($F_{7,49}$ = 16.44 and 71.70, P < 0.0001) in plasma corticosterone levels compared with controls in both male and female pups (Figure 7). These increases persisted throughout the period of nasal obstruction and decreased very slowly once nasal breathing restarted. There were no significant differences in corticosterone levels between control and obstructed males once into adulthood, but they were significant treatment differences in adult females: 14.13 ± 4.01 *vs* 42.77 ± 6.02 ng/ml, respectively ($F_{1,12}$ = 5.43, P < 0.006).

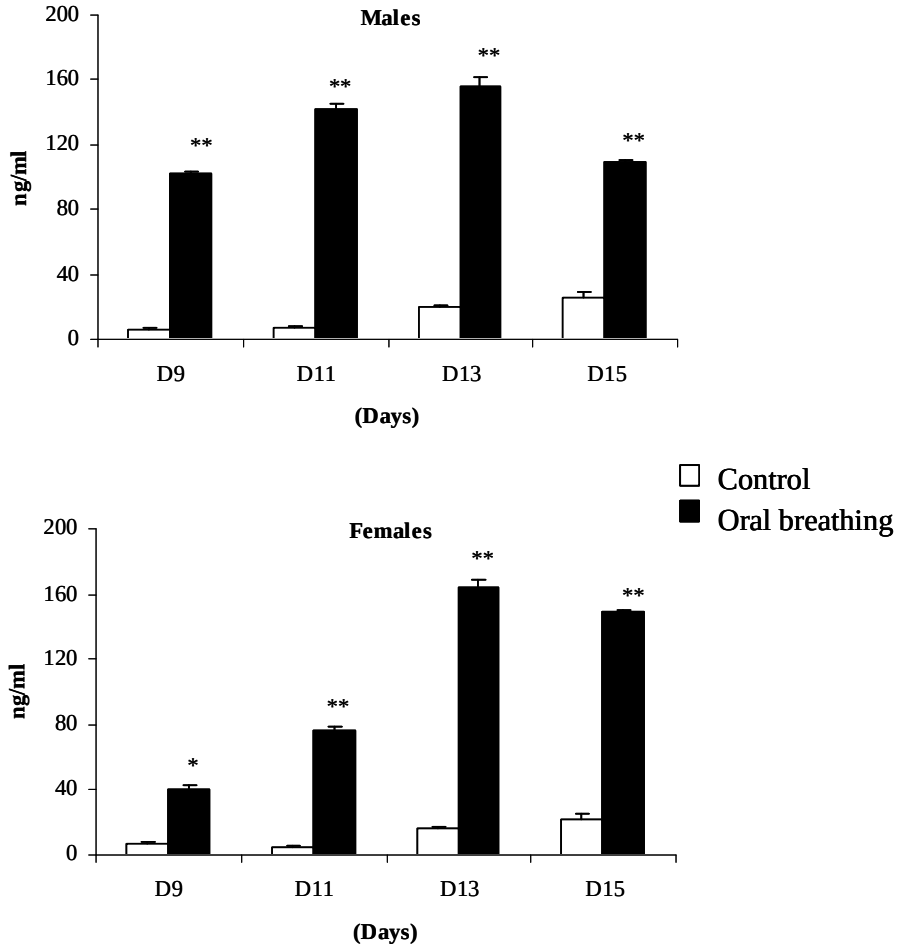

Figure 7. Impact of early nasal obstruction on plasma corticosteroid level at 9, 11, 13, and 15 days old in control and oral breathing animal. Values are means ± S.E.M (n = 7 rats/group/age). ANOVA summary: * significantly different from control group at P < 0.01; ** significantly different at P < 0.0001.

4 - Discussion

We have shown for the first time that a few days of forced oral breathing during reversible nasal obstruction induced body weight loss associated with dehydration (increased plasma osmolality) and increased release of vasopressin. As soon as nasal breathing resumed (around days 14 and 15) all parameters returned to control levels. Associated with these changes in hydration in response to nasal obstruction was an intense stress response in both male and female rat pups. The corticosteroid levels remained elevated on day15 when the signals of hydration had returned to normal. In adult males there were no significant differences in corticoid levels whereas in adult females the differences continued.

As already mentioned, there is an increased net water loss by oral compared to nasal respiration in healthy subject (Svensson *et al.*, 2006). In the present study, both male and female rat pups with nasal obstruction had small significant decrease in their gastric content during the days of oral breathing. In both male and female rat pups with nasal obstruction there was a significant increase in plasma osmolality and vasopressin release indicative of dehydration (Ji *et al.*, 2005; Parrott *et al.*, 1988). A significant increase in fluid loss appears to have been sufficient to cause a decrease in body weight. In both male and female oral breathing rat pups, the greatest increase in osmolality was recorded on the 3rd day after nasal obstruction. This is a very important point as body fluid regulation is very well controlled under normal conditions (Thornton, 2010). In the conditions of this experiment, the rats could not compensate for this dehydration. In rat pups before weaning feeding sessions would have been decided by the mother. The main recourse for the pups would have been to reduce urine loss, which is what would have happened with the increased vasopressin levels. Decreased urine production could have stimulated the mother to increase licking of the excretory organs to encourage urination. Fluid retention, by the anti-diuretic action of vasopressin, would have enabled the rat pups to regain body weight lost during the 3 to 4 days of nasal block once nasal breathing resumed between the 13th and 15th day measurements.

It would have been logical to have expected many empty stomachs during the first day after nasal obstruction as it would have been difficult for the rats to breathe and to eat/drink at the same time. Furthermore, the stomach contents were removed at a constant time in all the experiments and even in the controls we found some that did not contain any food. This could suggest that pups did not necessarily eat/drink at the same

time. However, there were more empty stomachs in the nasal obstruction groups, both male and female, than in control groups suggesting that oral breathers may have more difficulty feeding than the controls. This could explain the small, but significant, decrease in plasma glycemia on the first day of oral breathing in the females.

The first day of nasal obstruction appears to be a significant stress that stimulated a large release of corticosterone, especially in the oral breathing male pups. This is interesting in that the males had a smaller vasopressin response than the females. It is known that vasopressin and corticotrophin-releasing hormone (CRH) both play a synergistic role in stimulating the release of adrenocorticotropic hormone (ACTH) from the adenohypophysis (Walker, 1997) so it could be possible that in the pups vasopressin could have been used preferentially for enhancing the CRH effect during the first days of nasal obstruction-induced oral breathing. A similar stress effect has been observed previously by Gelhaye *et al.* (2006a) looking at facial muscle changes with oral breathing at weaning. This "stress"response could have been in response to the initial operation to block the nasal passage and/or the dehydration induced by the 2 to 3 days period of oral breathing. Water deprivation is a robust stress in rats (Ulrich-Lai & Engeland, 2002). The results of this work suggest that any event that produces short, or even long, term nasal obstruction, and thus forcing oral breathing, entails aspects of whole body dehydration. Under normal circumstances animals are able to compensate for this dehydration by increasing water consumption but we have not been able to find any data on this. It would be very interesting to investigate this point further.

In adult humans one of the hallmarks of OSA is multiple cycles of hypoxia/reoxygenation that appear to promote oxidative stree and inflammation (Lavie *et al.,* 2008) which could lead to cardiovascular disease, endothelial and metabolic dysfunction, and obesity (Foster *et al.,* 2009 ; Jun & Polotsky, 2009 ; Pack & Gislason, 2009 ; Jelic *et al.,* 2010). Our model of temporary nasal obstruction could be an appropriate model for looking at potential changes in hormones and other physiological parameters of obstructive sleep-disordered breathing. Furthermore, obstruction of the upper airway has been suggested to be an initiating factor in Sudden Infant Death Syndrome (Harding *et al.,* 1995) and dummy (pacifier) use to encourage nasal breathing has been suggested as a protective measure against this (L'Hoir *et al.,* 1999). These observations, as well as our present results, suggest that hydrational changes are extremely important to measure in cases of nasal obstruction and the breathing problems that result therein. Humans do not appear to be able to fully satisfy their

hydration needs (Bellisle *et al.*, 2010 ; Thornton, 2010) and this hypohydrated state may present further severe complications. Chronic dehydration has been suggested to exist in children with consequences on cognitive performance; increased water drinking in school age children increases cognitive performance (Edmonds & Burforf, 2009 ; Edmonds and Jeffes, 2009). If this is the normal situation then the cognitive problems encountered in children with sleep-disordered breathing (Rombaux *et al.*, 2005 ; Armengot *et al.*, 2008 ; Craig *et al.*, 2008) would be further exacerbated.

In conclusion, a short period of forced oral breathing following reversible nasal obstruction produces temporary dehydration (increased osmolality and vasopressin release) with decreased body weight gain. Both recover to control levels once nasal breathing begins. Oral breathing produces an obvious stress response which appears to outlast the return to normal of the other physiological parameters. The present procedure may serve as a model of human conditions involving forced oral breathing that produce several deleterious effects in children and adults.

CHAPITRE II

Incidences d’une obstruction nasale précoce sur le développement physiologique et morphologique.

Nous venons de montrer que le passage à la respiration orale est associé à une **perte hydrique** importante chez les jeunes rats et que l'obstruction nasale est associée à la mise en place d'une suppléance buccale afin de maintenir la fonction ventilatoire. Le schéma dysfonctionnel de la respiration orale constitue une réaction d'adaptation en chaîne. La respiration orale entraîne une augmentation de l'activité des muscles respiratoires accessoires (scalènes et pectoraux). Ce syndrome est maintenu par la baisse de l'activité du diaphragme et de l'hypotonie des muscles abdominaux (Lima *et al.,* 2004). Les patients atteints du syndrome de respiration orale, ont une plus grande activité des muscles inspirateurs accessoires, ce qui entraîne une augmentation de la consommation énergétique et une mauvaise ventilation pulmonaire (Ribeiro *et al.,* 2002). Les patients développent également une hypertrophie des muscles inspirateurs par manque de synergie avec les muscles abdominaux (Hruska, 1997)

L'obstruction nasale est donc une situation multifactorielle occasionnant de nombreuses conditions capable de modifier le développement des muscles respiratoires, et plus particulièrement une adaptation des muscles oro-faciaux qui représentent une unité fondamentale de la respiration nasale active, comme les flairages.

L'absence de flairage et l'ouverture chronique de la bouche constituent des variations de conditions de travail susceptibles de modifier de manière spécifique l'activité des muscles oro-faciaux. Ainsi, alors que l'obstruction nasale inhibe l'activité électromyographique du masséter chez le chat, l'activité électromyographique du diaphragme n'est pas affectée (Ono *et al.*, 1998). De la même façon, l'activité du muscle *orbicularis oris,* impliqué dans la fermeture des lèvres, est inhibée lors du passage à la respiration buccale (Song & Pae, 2001). Ces résultats suggèrent que la respiration buccale inhibe l'activité électromyographique des muscles responsables de la fermeture de la mâchoire. À l'inverse, à travers l'activation de mécanorécepteurs, le passage à la respiration buccale stimule l'activité électromyographique des muscles de l'oropharynx tels que les muscles

supra-hyoïdiens, facilitant ainsi la respiration (Hiyama *et al.*, 2003). La respiration buccale entraîne également une augmentation de l'activité du muscle *levator palatini*, élévateur du voile du palais, et une diminution de l'activité du muscle palatoglosse qui isole la cavité buccale des voies aériennes supérieures en se contractant (Tangel *et al.*, 1995). Enfin, chez l'Homme en exercice, le passage à la respiration buccale inhibe l'activité du muscle dilatateur des ailes du nez tandis que l'activité du muscle genioglosse, dépresseur de la langue, n'est pas affectée (Shi *et al.*, 1998; Williams *et al.*, 2000). L'absence de respiration nasale engendre donc d'importantes modifications de l'activité électromyographique des muscles des voies aériennes supérieures, ces variations agissent de manière synergique de façon à libérer une voie aérienne buccale permettant de maintenir la pérennité de la fonction ventilatoire.

1 – Structure et fonctions des muscles squelettiques

Les muscles squelettiques présentent une grande diversité de performances et de spécialisations, liée principalement à la diversité des fibres qui les composent. Ils renferment en effet plusieurs types de fibres qui diffèrent par leur structure, leur métabolisme, leur puissance et leur vitesse de contraction. On distingue ainsi des fibres à **contraction lente** (fibres de type I) et des fibres à **contraction rapide** (fibres de type II - Bottinelli *et al.*, 1991). La fibre musculaire constitue l'unité de base du muscle strié, elle exprime les protéines impliquées dans la contraction et les enzymes indispensables au métabolisme énergétique. Les protéines contractiles présentent pour la plupart un polymorphisme. Parmi celles-ci, la **myosine**, protéine majoritaire du muscle, existe sous plusieurs isoformes et chaque isoforme contient deux chaînes protéiques de hauts poids moléculaires (200 à 220 kDa) appelées **chaînes lourdes de myosine** (**MHC**).

Chez les Mammifères, les MHC sont divisées en huit classes principales. Leurs expressions respectives varient en fonction du type de muscle et du stade de développement considéré (Pette & Staron, 2000). Les quatre isoformes principales de MHC discernables dans les muscles squelettiques du rat adulte correspondent à trois de **types "rapides"**, MHC_{2a}, MHC_{2x} et MHC_{2b}, et à un de **type "lent"** MHC_1 (Bär & Pette, 1988; Schiaffino *et al.*, 1989). Les fibres à MHC_{2b} présentent la vitesse de contraction la plus élevée, suivies des fibres à MHC_{2x}, des fibres à MHC_{2a}, puis des fibres à MHC_1 (Bottinelli *et al.*, 1991). Bien que les mécanismes

à l'origine de ces différences soient encore mal compris, ceux-ci impliquent sans doute des variations dans le turnover de l'ATP, c'est-à-dire dans le rapport entre la production et la consommation d'ATP : les **fibres de type II** génèrent plus rapidement l'ATP, grâce à leur activité glycolytique et du fait d'une cinétique d'activation de l'ATP_{ase} supérieure à celle des **fibres de type I** (Sant'Ana Pereira *et al.*, 1996; Polla *et al.*, 2004). Par conséquent, les fibres de type II sont adaptées aux contractions rapides et puissantes mais manquent en revanche d'endurance. Les fibres de type I, moins puissantes mais plus endurantes, sont caractéristiques des muscles posturaux et du diaphragme. Sieck *et al.*, (1996) ont ainsi classé les unités motrices en quatre types principaux :

- Fibre I (**MHC_1**) : contraction lente et résistante à la fatigue
- Fibre IIA (**MHC_{2a}**) : contraction rapide et fatigabilité intermédiaire
- Fibre IIX (**MHC_{2x}**) : contraction rapide et peu résistante à la fatigue
- Fibre IIB (**MHC_{2b}**) : contraction rapide et peu résistante à la fatigue

Ainsi, la grande variabilité de la myosine représente l'un des principaux facteurs de polymorphisme des fibres musculaires (Pette & Staron, 2000). L'abondance relative des différents types de fibres détermine les caractéristiques physiologiques des muscles, c'est-à-dire détermine, si un muscle est plus ou moins fatigable (Sieck *et al.*, 1996; Geiger *et al.*, 1999, 2000).

La différenciation des fibres musculaires commence avant la naissance et la mise en place des propriétés contractiles s'effectuent progressivement au cours du développement (Usami *et al.*, 2003). Les **muscles embryonnaires et néonataux** diffèrent des **muscles adultes** par leurs propriétés contractiles et leurs compositions en isoformes de myosine (Nelson & Thompson, 1994). Les fibres néonatales les plus lentes contiennent de la **MHC_1**, les fibres intermédiaires possèdent de la **MHC_e** (embryonnaire), de la **MHC_p** (périnatale) et de la **MHC_{2a}**, et les fibres les plus rapides contiennent de la **MHC_p** et de la **MHC_{2a}**. La présence de **MHC_{2x}** et **MHC_{2b}** a néanmoins été décrite au cours du développement périnatal dans certains muscles tels que le diaphragme et le masséter superficiel (Zhan *et al.*, 1998; Usami *et al.*, 2003). La mise en place des caractéristiques contractiles des fibres s'effectue progressivement au cours du développement, plus ou moins rapidement selon les espèces. Le programme de développement de l'expression des différentes isoformes dépend non seulement du type de muscle, mais également des conditions hormonales et environnementales (Whalen *et al.*, 1981). Dans tous les cas, la structure de base et la proportion relative de chaque type

de fibre se diversifient après la naissance, permettant l'adaptation de la musculature à la posture et aux mouvements. Les muscles de rat présentent ainsi un phénotype immature à la naissance avant d'exprimer des phases de transition entre les différentes isoformes de MHC selon le schéma général suivant (Pette & Staron, 2000; White *et al.*, 2000) :

$$MHC_e \rightarrow MHC_p \rightarrow MHC_1 \leftrightarrows MHC_{2a} \leftrightarrows MHC_{2x} \leftrightarrows MHC_{2b}$$

2 – Obstruction nasale et morphologie des muscles oro-faciaux

L'obstruction nasale et la respiration buccale associée, entraînent des modifications de l'activité électomyographique des muscles des voies aériennes supérieures. De telles modifications sont connues pour influencer la masse musculaire (Roy *et al.*, 1991). Ainsi, une stimulation électrique quotidienne prévient l'atrophie liée à l'immobilisation chez le lapin (Qin *et al.*, 1997). L'hyperactivité entraîne par ailleurs des transitions vers des isoformes de MHC plus lentes mais plus résistantes à la fatigue (Asmussen *et al.*, 2003). Une activation ou une stimulation électrique de longue durée accroît ainsi la proportion des fibres de type I au détriment des fibres de type II (Delp & Pette, 1994; Windisch *et al.*, 1998). De plus, une corrélation a été démontrée entre la quantité d'activité musculaire quotidienne et la proportion de fibres de type I (Kernell *et al.*, 1998). À l'inverse, on constate des transitions vers des fibres à contraction rapide lors de périodes d'activité réduite (Edgerton *et al.*, 1995). En cas de dénervation, on observe une diminution des concentrations relatives en MHC_1 et MHC_{2b} et une augmentation concomitante en MHC_{2a} et MHC_{2x} (Huey & Bodine, 1998; Jakubiec-Puka *et al.*, 1999). Ainsi, en l'absence d'innervation, les muscles à contraction rapide deviendraient plus endurants et les muscles posturaux deviendraient moins résistants à la fatigue (Pette & Staron, 2000). Les transitions ne dépendent donc pas uniquement de l'activité neuromusculaire, mais également des propriétés intrinsèques aux fibres musculaires. De plus, ces transitions peuvent être facilitées, ou au contraire modulées, par le contexte hormonal (Kelly & Goldspink, 1982; Kelly *et al.*, 1985; Butler-Browne *et al.*, 1990).

Les modifications spécifiques de stimulation électrique sont donc susceptibles d'avoir un effet sur l'expression des isoformes de MHC. Etant donné que la composition en MHC détermine les propriétés contractiles des muscles, des variations

dans la répartition en isoformes de MHC pourraient être impliquées dans l'adaptation à la respiration buccale.

Nos résultats (cf. article 3), tout comme ceux de Schlenker et al. en 2000 et Gelhaye en 2007, montrent que suite à l'obstruction des narines, il y a mise en place d'une suppléance buccale, afin de maintenir la fonction ventilatoire. La respiration buccale nouvellement établie, se traduit par un rythme d'ouverture qui augmente progressivement jusqu'au 11ème jour postnatal, et qui diminue ensuite de façon graduelle, avant de chuter brutalement à J15, ce qui correspond au jour de la réouverture des narines.

Deux facteurs non exclusifs doivent être pris en compte pour expliquer les modifications observées entre le 8ème et le 14ème jour postnatals : la maturation des mécanismes permettant l'établissement d'une voie aérienne buccale et le degré de détresse respiratoire engendré par l'obstruction de la filière nasale. En effet, les études chez la brebis montrent qu'en cas d'obstruction nasale, les perturbations de l'homéostasie gazeuse du sang sont plus marquées chez le nouveau-né (Harding *et al.*, 1987). Ce résultat peut être expliqué par une immaturité des mécanismes nerveux permettant l'établissement et le maintien de la respiration buccale (Miller *et al.*, 1985; Harding & Wood, 1990). La capacité à maintenir une voie aérienne buccale augmente ainsi avec l'âge du fait de la maturation des chémorécepteurs sanguins d'une part, et des mécanorécepteurs des voies aériennes d'autre part (Harding *et al*., 1995). De plus, l'obstruction des narines entraîne une détresse respiratoire qui pourrait atteindre son maximum le 11ème jour postnatal. Cette hypothèse est notamment soutenue par les travaux montrant que chez le rat adulte, les modifications de l'homéostasie gazeuse du sang s'aggrave dans les 72 h suivant l'obstruction des narines (Erkan *et al.*, 1994).

Nos résultats (cf. article 3), montrent que les mouvements mandibulaires liés à la respiration buccale, suite à l'absence de respiration nasale, entrainent des modifications de conditions de travail, influençant le développement des muscles oro-faciaux dès le 1er jour (J9) qui suit l'obstruction nasale. Ainsi, l'obstruction nasale et l'ouverture chronique de la bouche entraînent des modifications de la répartition en isoformes de MHC dans tous les muscles oro-faciaux étudiés chez les mâles au stade

néonatal (J9 et J11) et à l'âge adulte (J90). De nombreux facteurs sont susceptibles d'influencer le développement musculaire suite à l'induction d'une obstruction nasale expérimentale. En modifiant de façon spécifique l'activité électromyographique des muscles oro-faciaux, les variations de conditions de travail constituent sans doute le facteur déterminant les changements muscle-spécifiques constatés dans notre étude. De plus, l'obstruction nasale est susceptible d'entraîner de nombreuses conditions pouvant affecter de manière systémique les muscles squelettiques. Ainsi, la privation alimentaire et les modifications endocriniennes, observées pendant la période de l'obstruction nasale, sont connues pour leurs effets sur le développement du système musculaire. Ces facteurs peuvent favoriser ou, au contraire, contrecarrer les effets spécifiques liés aux modifications de conditions de travail.

En outre, le statut hormonal des individus est sans doute impliqué dans les modifications structurelles observées suite à l'induction de l'obstruction nasale.

Nos résultats montrent que l'obstruction nasale et la respiration buccale forcée chez les jeunes, sont associées à des niveaux plasmatiques élevés de corticostérone et de testostérone, et à des niveaux faibles d'hormones thyroïdiennes.

Les corticoïdes ont un effet catabolique sur les muscles squelettiques (Seene & Viru, 1982). Cet effet est lié à une diminution de la synthèse des protéines myofibrillaires, à une augmentation de leur catabolisme et à une réduction des stocks protéiques (Seene *et al.*, 2003). Ces hormones produisent donc des changements spécifiques dans l'expression des isoformes de MHC (Kelly & Goldspink, 1982) et cet effet spécifique pourrait expliquer les modifications des MHC dans le **masséter superficiel** et le **digastrique antérieur**.

Les hormones thyroïdiennes jouent par ailleurs un rôle essentiel dans le développement des muscles squelettiques (d'Albis *et al.*, 1990). Elles stimulent par exemple la transition des isoformes neonatales vers les isoformes adultes en anticipant l'expression de la MHC_{2b} au cours du développement postnatal (Butler-Browne *et al.*, 1990). Or l'hypothyroïdisme entraîne un retard de maturation du masséter superficiel qui présente alors une réduction du diamètre des fibres musculaires (Ganji & Behzadi, 2007).

L’augmentation du taux de testostérone peut être en relation avec l’augmentation des soins maternels que nous avons vu dans l’article 1.

En résumé, la réduction du niveau d’hormones thyroïdiennes et l’augmentation du niveau de corticostérone constatées chez les animaux du groupe obstruction nasale, pourraient expliquer les transitions vers des isoformes plus résistantes à la fatigue observées au niveau des muscles liés à la respiration buccale dans notre étude (cf. article 3).

Enfin, nos résultats sur les mâles, par rapport à ceux obtenus sur les femelles de 21 jours par Gelhaye *et al.*, (2006a), montrent que la structure musculaire des muscles oro-faciaux diffère en fonction du sexe des individus. Il s’agit d’un résultat peu surprenant dans la mesure où les muscles masticatoires de plusieurs espèces de Mammifères sont sexuellement dimorphiques à l’âge adulte du point de vue de leurs compositions en MHC. C’est par exemple le cas chez le cochon d’inde (Lyons *et al.*, 1986), le lapin (English *et al.*, 1999), la souris (Eason *et al.*, 2000) et le macaque rhésus (Maxwell *et al.*, 1979). Ainsi dans le masséter superficiel, le dimorphisme sexuel est inversé suite à l’induction de l’obstruction nasale : contrairement à ce qui est observé chez les individus témoins et contrôles, les mâles expérimentaux présentent plus de MHC_{2b} que leurs homologues femelles.

3 – Obstruction nasale et développement du crâne

Le **crâne** est une structure osseuse ou cartilagineuse de la tête, caractéristique des crâniates (dont font partie les vertébrés). Le rôle principal du crâne est de protéger le système nerveux central qu'il entoure, il sert aussi de points d'attache à des muscles du visage et du cou.

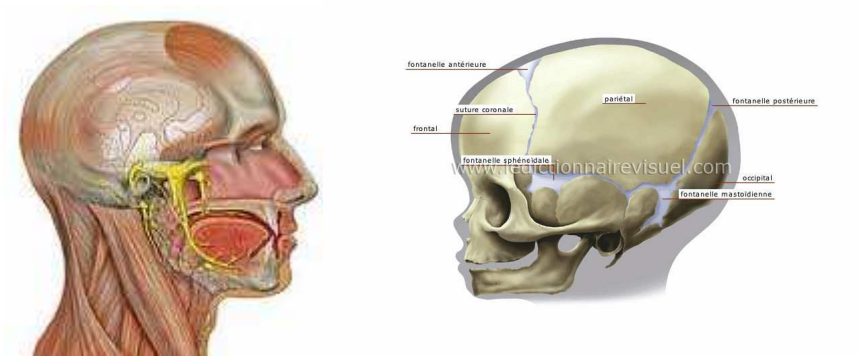

Figure 8 : Structure et organisation de la boitte crânienne

Le crâne est composé de deux parties : le **neurocrâne** (ou boîte crânienne), partie supérieure qui protège le cerveau et les organes sensoriels pairs, et le **splanchnocrâne** (ou viscérocrâne, ou crâne facial), partie inférieure qui soutient les cavités buccales et pharyngiennes. Le splanchnocrâne a primitivement une fonction respiratoire mais cette fonction se perd dans la plupart des lignées. Le splanchnocrâne entre donc principalement dans la constitution du squelette des mâchoires, du palais, de l'oreille moyenne, de la face, du cou, de la langue, du larynx et du neurocrâne.

Certaines espèces possèdent aussi un **dermocrâne**, formé d'os dermiques, qui recouvre le neurocrâne et le splanchnocrâne. Le crâne est toujours cartilagineux chez l'embryon mais devient, au moins partiellement, ossifié chez l'adulte pour toute la lignée des Ostéichtyens (qui inclut l'Homme).

Il est actuellement incorporé aux deux formations précédentes tout en conservant une action inductrice sur le développement de certains os dermiques (voûte du crâne, voûte palatine, différentes unités du splanchnocrâne).

Histologiquement, le crâne des Vertébrés peut passer par différents stades.

* Un stade cartilagineux ou **chondrocrâne**, formé par chondrification du mésenchyme. Ce crâne est définitif chez les lamproies et les Chondrichthyens,

* Un stade osseux ou **ostéocrâne endosquelettique** (ou enchondral), issu d'une ossification enchondrale. Ce stade succède au chondrocrâne chez tous les Ostéichthyens à l'état adulte.

* Certaines espèces possèdent aussi un **ostéocrâne exosquelettique** (ou dermocrâne, ou dermatocrâne), formé d'os dermiques (ou os membraneux). Cette ossification dermique se fait directement depuis le tissu conjonctif sans passer par un stade cartilagineux.

* Neurocrâne et splanchnocrâne sont issus de deux mécanismes embryologiques différents. Le neurocrâne est issue du mésenchyme. Ces éléments peuvent se présenter sous deux formes : une cartilagineuse qui forme le crâne cartilagineux ou **chondrocrâne** (crâne définitif chez les Chondrichtiens, les Myxine et les Lamproie). Chez tous les autres vertébrés cette forme est remplacée par une forme osseuse qui donne l'**ostéocrâne**. Chez les mammaliens le **chondrocrâne** (ou neurocrâne) se forme à la base du crâne embryonnaire et se compose de trois noyaux cartilagineux: le **basi-ethmoïde**, le **basi-sphénoïde** et le **basi-occipital**. Les cartilages paracordaux se développent pour former la plaque basale et d'autres cartilages occipitaux se développent pour former l'arc occipital (cartilage entourant complètement la corde). Des cartilages vont être associés à la plaque éthmoïde (issu du développement de cartilages trabéculaires) : ce sont les **capsules olfactives** et les **capsules nasales**. La moelle épinière se dilate dans le chondrocrâne en passant dans le foramen magnum. L'oreille interne occupera la **capsule otique**.

Pendant la vie embryonnaire, le tissu cartilagineux forme temporairement la plus grande partie de l’échauche squelettique. C’est en son sein que se développe le processus d’ossification endonchondrale qui régit l’ossification de ces ébauches et la formation des os. Le cartilage de croissance s’ossifie après la naissance, à l’exception du **septum nasal** chez les rongeurs et chez l’homme, qui persiste jusqu’à l’âge adulte. Le septum nasal se développe au centre de la face et sa structure est impliquée dans la morphogenèse, le développement et la croissance du massif facial. Il se développe jusqu’à l’âge adulte par ossification endochondrale d’une manière identique chez le rat, la souris, le lapin et l’homme.

Chez l’homme, l’obstruction nasale néonatale associée à la respiration orale entraîne des troubles de la croissance du tiers moyen de la face (Rombaux *et al.,* 1998). En effet, le mode respiratoire est susceptible d’influer sur la position de la mandibule et de la langue, on observe ainsi la mise en place d’une béance labiale (position basse de la

mandibule) de façon à établir une voie aérienne buccale (Rubin, 1980, Shikata *et al.*, 2004). A long terme, ces modifications induisent des troubles généralement irréversibles de l'articulé dentaire, avec la formation d'un palais étroit et ogival pouvant aller jusqu'à des problèmes de malocclusion et un surdéveloppement vertical de la face (Schlenker *et al*., 2000 ; Crouse *et al.*, 2000). Ces symptômes sont plus fréquents chez les enfants en âge pré-scolaire (Peltomäki 2007 ; Juliano *et al*., 2009).

Chez les rongeurs, le développement du crâne et de la région naso-maxillaire est très complexe. Le développement de la cavité nasale présente un schéma de croissance différent de celui du crâne. La croissance de l'os nasal est observée dans les surfaces extérieures et la résorption osseuse se fait dans les surfaces internasales. La croissance des sutures est très active et varie en fonction de l'os présent de chaque côté de la suture (Youssef, 1966).

Nos études, conduites sur les rats pendant la période de fermeture totale des narines et 90 jours après la réouverture (cf. article 4), montrent que l'obstruction associée à la respiration buccale entraîne un ralentissement du développement du complexe naso-maxillaire, et de la longueur totale du crâne dans les deux sexes dès les premiers jours. Ces résultats sont maintenus à long terme chez les animaux, sauf pour le complexe naso-maxillaire des femelles, en effet celui-ci se développe de manière comparable à celui des contrôles. L'obstruction nasale entraîne une croissance réduite des bulbes olfactifs, et également des poumons. A l'âge adulte, les bulbes olfactifs sont significativement moins développés chez les rats expérimentaux par rapport aux rats contrôles, alors que la taille des poumons est comparable.

Article 3

Effects of short term forced oral breathing: physiological changes and structural adaptation of diaphragm and orofacial muscles in rats

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Archives of Oral Biology (sous presse)

1. Introduction

Evidence is now available showing that muscle contractile properties and myosin heavy chain (MHC) composition are correlated (Bottinelli *et al*., 1996). The four major myosin heavy chain isoforms detectable in adult skeletal muscles are three fast types, MHC 2a, 2x and 2b, and one slow type, MHC 1 (Bär & Pette, 1988). During development, two perinatal MHC forms can be found in muscle fibers depending on the stage considered: MHC embryonic and neonatal. These different MHC appear sequentially in fast and slow muscles and the developmental program of myosin expression depends greatly on the type of muscle (d'Albis *et al.*, 1989). Thus, at birth, rat muscles are phenotypically immature and during development, or when the working conditions are changed, marked transitions in the myosin content can occur in rat fast and slow-twitch muscles (Swynghedauw, 1986 ; Geiger *et al.*, 2006). These modifications generally adapt the muscle to the new environmental requirements (Grunheid *et al*., 2009).

Chronic nasal obstruction is a non-specific condition observed in many pathological conditions, e.g. rhinitis. Nevertheless, because this disorder is not life threatening (at least in adults) its importance could be underestimated. Impaired nasal breathing results in obligatory oral breathing, which can to be divided into two components: chronic absence of active nasal respiration that results in an olfactory deprivation (Meisami, 1976), and chronic mouth opening (Schlenker *et al.*, 2000). Furthermore, in contrast to oral breathing, nasal breathing allows the optimal

conditioning of inhaled air, clearing, moistening and warming the air before the gas exchange in the lungs (Betlejewski, 1998 ; Keck *et al.,* 2001). Thus, nasal obstruction could be associated with both social (maternal behaviour, relation with congeners) and physical (environmental privation, respiratory modification) stress. In other words, it is possible that nasal obstruction causes a loss of the sense of smell and this hyposmia could disrupt the orientation of young rats to the mother, with consequent deprivation of food and feeding. It has been shown in rats that deprivation of food for 3 days causes a diminution in thyroid hormones (Laws *et al*., 2007), and other studies have also shown that increased mother licking of the pups is needed for testosterone production necessary for masculinisation of the young male rat (Moore *et al.,* 1992).

Stressful situations correspond to particular changes in environmental conditions that induce modifications in different physiological parameters like plasma hormonal levels. For example, stressful situations produce an adrenal hypertrophy and an increase of plasma glucocorticoid levels (Basset & West, 1997 ; Gomez *et al.,* 1996), which are known to induce alterations in MHC isoforms expression (Polla *et al.,* 1994).

Plasma levels of thyroid hormones can be reduced in stressful situations and these hormones are very important in the normal development of vertebrate skeletal muscle, notably in muscle MHC distribution (d'Albis *et al.,* 1990). For example, thyroid hormones are known to stimulate the transition from neonatal type to adult type and to anticipate the expression of MHC 2b during post-natal development (Butler-Browne *et al.,* 1990). Thus, reduced thyroid hormone levels and increased EMG activity could explain the fast-to-slow transitions found in muscles related to mouth opening in oral breathing animals, but this remains to be confirmed.

In addition, the contribution of orofacial muscles to the variation in bite force magnitude is correlated with craniofacial morphology (Raadsheer *et al.,* 1999), and chronic oral breathing is known to be a contributing factor in deviant facial growth patterns in pre-school children (Mattar *et al.,* 2004 ; Yang *et al.,* 2002). These patterns are the result of a prolonged presence of unbalanced oro-pharyngeal muscle activity. Through mechanoreceptors, oral breathing stimulates oro-pharyngeal electromyographic activity of the muscles facilitating respiration (Song & Pae, 2001). These modifications of electric stimulation may have an effect on MHC isoform

expression (Pette & Vrbova, 1999), and the MHC isoform composition of orofacial muscle could be involved in the adaptation to oral breathing during nasal obstruction. In a previous study, Gelhaye *et al.* (2006a) showed that nasal obstruction induced by external cauterisation of the nostril, in 8 day old rat female pups, produced total nasal obstruction during the next 4 days followed by gradual reopening of the nostrils, complete at 15 days. Furthermore, these authors found that this nasal obstruction was associated with chronic oral breathing. At weaning (day 21), the only period studied, the chronic oral breathing animals presented an atrophy of olfactory bulbs, hypertrophy of the adrenal glands and reduced muscle growth for all muscles studied except for the diaphragm. A decrease of MHC 2b compared to MHC 2a in *levator nasolabialis*, a muscle involved with nasal breathing in the oral breathing group was observed. In *masseter superficialis* and *anterior digastric*, muscles involved with oral breathing, an increase of MHC 2b in *masseter superficialis* and a decrease of MHC 2a in *anterior digastric* to the benefit of MHC 2x were detected. No significant difference was detected at day 21 (D21) in diaphragm MHC expression in oral breathing animals.

To our knowledge no work has been published of MHC changes during short term reversible nasal obstruction on male rats. Thus, our hypothesis was that nasal obstruction would have a significant effect of MHC isoform expression of muscles involved in breathing including the diaphragm during the very short period of forced oral breathing. We investigated also if these changes were maintained over the long term, *ie* up to adulthood, day 90 (D90). The effect of early nasal obstruction on various organ weights, on the stress response and on plasma levels of thyroid hormones (T3 and T4) and androgens (testosterone) was also studied.

2. Materials and Methods

2.1. Animal care

Forty two male Wistar rats (IFFA-CREDO, France) were used for this experiment. The animals were born in the laboratory from twenty litters, culled to 7 pups per litter to ensure normal body growth. The animals were housed in standard

cages under controlled temperature conditions (22 ± 1°C). Food and water were available *ad libitum* throughout the experiment. From birth, the rats were kept on a reversed 12:12 light-dark cycle (dark period 08:00 - 20:00).

2.2. Nasal obstruction procedure

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (no. 85-23, revised 1996), the recommendations of the European Community Council for the Ethical Treatment of Animals (no. 86/609/EEC) and the regulations of the University of Nancy 1. All efforts were made to minimize animal suffering.

At the age of 8 days (D8), the litters were first anesthetized. Animals were weighed and they were then divided randomly into one control group and one experimental group (oral breathing). Bilateral nasal obstruction, resulting in forced oral breathing, was performed in experimental animals (7 per age) as described previously by Meisami (1976), and Waguespack *et al.* (2005). The selected method consisted in the cauterization of the external nostrils, which is the most common and simple procedure allowing spontaneous reopening of nostrils after 4 days. The tissue surrounding the external nostrils was burned by placing a surgical cauterizing instrument (1 mm in diameter) on the nostrils, consequently occluding the orifice of the nostrils without mechanical or chemical damage to the olfactory mucosa. This procedure induced complete nasal obstruction between D8 and day 11 (D11) with 100 % of the nostrils spontaneously reopened by day 15 (D15). The sampling experiments were conducted during complete nasal obstruction day 9 (D9) and day 11 (D11) and at 90 days after post-reopening of the nostrils, ie at the beginning of adulthood.

The animals started breathing through their mouths immediately after nasal occlusion, as has been reported in infants (de Almeida *et al.*, 1994). Nostril cauterisation earlier in life resulted in rapid death of the pups.

In the control group (7 per age), the nostrils were not sealed but the cauterizing instrument was placed about 1-2 mm above each nostril to burn the skin. After cauterization, the nostrils were washed with chlortetracycline (Aureomycine Evans 3%)

to prevent infection. The pups were warmed (37°C) for 30mn and returned to their mothers.

Exploratory and feeding behaviours of the pups after weaning were the same for both cauterised and control group rat pups suggestive of no serious long term central effects of the treatment, especially in the forced oral breathing group (Gelhaye *et al.*, 2011).

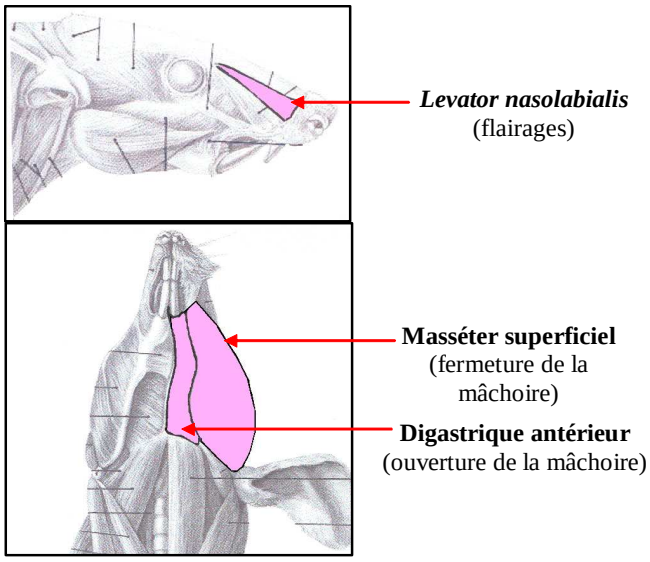
2.3. Sample collection

Seven male rats per group (control and oral breathing) and per age (D9, D11 and D90), were removed, immediately humanely killed, weighed and an intracardiac blood sample (500 – 1000 µl) was taken between 11h and noon for hormonal measurements. Blood was collected over 1-2 min into sterile heparinised syringes fitted with a 26-G needle. Plasma was immediately separated from cells by centrifugation (4°C, 15 min at 3000 rpm) and the extracts were stored at -18°C until assayed.

After blood sampling, olfactory bulbs, lungs, testicles and adrenal glands were removed bilaterally and weighed. Adrenal weight is a direct indicator of chronic stress exposure (Basset & West, 1997).

2.4. Muscle sampling and myosin extraction

After sample collection, the entire Diaphragm (Dia, respiratory muscle) was dissected, and the following muscles removed unilaterally (right hand side): *Anterior Digastric* (AD, depressor mandibular muscle), *Masseter Superficialis* (MS, propulsive mandibular muscle) related to mouth movements and oral breathing (Spyropoulos *et al.*, 2002 ; Van Wessel *et al.*, 2005), and *Levator Nasolabialis* (LN, active sniffing muscle) related to nasal breathing (Hartmann *et al.*, 1999). After dissection, muscles were weighed and myosin was isolated in a high ionic strength buffer, as described by d'Albis *et al.* (1979).



2.5. Electrophoretic analysis of myosin heavy chain isoforms

Electrophoresis was performed according to the method of Talmadge & Roy (1993) with little modification. This allowed the separation of the developmental MHC as described by Janmot and d'Albis (1994), and Ohnuki *et al.* (2009). Mini-gels were used in the Bio-Rad Mini-protean II Dual Slab Cell. Electrophoresis took place in a cold room (temperature of 6 °C) for the whole run. To separate all the heavy chains, the duration of the run was 32 h (70 V) for animals aged D9 and D11, and 28 h (70 V) for adults (D90). Three separate loads were made per sample (2.5 µg of protein/well). The MHC isoforms were identified according to migration rates compared with an adult diaphragm containing only adult isoforms 2a, 2x, 2b and 1 (Pette & Staron, 1990). The gels were stained with Coomassie blue R-250. The relative amounts of the different myosin heavy chains were measured using an integration densitometer Bio-Rad GS-800 and analyzed with the Molecular Analyst Program (Quantity One 4.2.1).

2.6. Hormone assays

Corticosterone and **testosterone** concentrations were measured without an extraction procedure, using a commercially available EIA kit and performed according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentration of corticosterone and testosterone in plasma samples was calculated from a standard curve

and expressed as ng/ml. The intra- and inter-assay coefficients of variation were under 8.4% and 13.1%, respectively for corticosterone, 10.8% and 14.6% respectively for testosterone.

Thyroxine (T4) and **triiodothyronine** (T3) were assayed using commercial RIA kits and performed according to the manufacturer's guidelines (Immunotech SA, Marseille, France). The concentrations of T4 and T3 in plasma samples were calculated from standard curves and expressed as pg/ml. The intra- and inter-assay coefficients of variation were respectively under 6.7 and 6.5 % for T4 and under 6.4 and 5.5 % for T3.

2.7. Statistical analysis

The results were expressed as group means ± SEM. Student's t-test was used to establish the comparison between control and oral breathing animals since all data were normally distributed. Body weight group differences were determined using a two-way ANOVA (factor treatment × factor age). Specific mean comparisons were then made using t-test with the Bonferroni correction. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Morphometric characteristics

Before treatment, at 8 days of age, the body weights of control and oral breathing pups were not significantly different: 17.78 ± 0.52 g and 17.95 ± 0.28 g, respectively ($t = 2.80$, $P = 0.14$). Table 1 shows that there was a significant difference in body weight already at D9 ($t = 4.45$, $P < 0.0001$) which continued on D11 ($t = 3.23$, $P = 0.002$) between control and oral breathing rats. No differences in body weights were observed at D90 ($t = 1.11$, $P = 0.28$).

	9 days	11 days	90 days
Control group			
Body weight (g)	18.60 ± 0.49	23.08 ± 0.59	408.47 ± 9.61
Olfactory bulbs (mg/g)	1.62 ± 0.13	1.21 ± 0.03	0.22 ± 0.01
Lungs (mg/g)	20.60 ± 0.52	18.83 ± 0.54	4.69 ± 0.14
Adrenal glands (mg/g)	0.34 ± 0.03	0.32 ± 0.01	0.19 ± 0.01
Testicles (mg/g)	2.11 ± 0.07	2.66 ± 0.11	7.55 ± 0.22
Oral breathing group			
Body weight (g)	15.51 ± 0.48*	19.75 ± 0.79*	394.18 ± 8.65
Olfactory bulbs (mg/g)	1.12 ± 0.12*	0.88 ± 0.04*	0.13 ± 0.01*
Lungs (mg/g)	20.61 ± 1.12	16.20 ± 0.02*	4.50 ± 0.36
Adrenal glands (mg/g)	0.41 ± 0.01*	0.49 ± 0.03*	0.23 ± 0.01*
Testicles (mg/g)	2.24 ± 0.05	2.65 ± 0.10	7.95 ± 0.24

Table 1. Effects of temporary forced oral breathing on body weight (g), olfactory bulb, lung, adrenal gland and testicle weights (mg/g) at age 9, 11, and 90 days in controls and animals exposed to 4 days of nasal obstruction. Values are means ± S.E.M (n = 7 / group / age). Analysis of two-way ANOVA summary: age effect: F = 105.44 to 4584.50 at two degrees of freedom P < 0.0001; treatment effect: F = 5.17 to 7.04 at one degrees of freedom P = 0.02 to < 0.0001; age ^{x} treatment: F = 6.18 to 11.86 at two degrees of freedom P = 0.005 to 0.0001. Analysis of t-test with Bonferroni correction: *significantly different from control group at 9, 11 and 90 days at P < 0.05.

The body weight of animals decreases in 14 % at D9 in the oral breathing group compared to weights at D8 (respectively 15.51g *vs* 17.95g) and also 14 % compared to control at D11 (respectively, 19.75g *vs* 23.08g).

Relative organ weights are presented in Table 1. Olfactory bulb weights: a significant reduction in olfactory bulb weight was found for the three ages in the oral breathing group compared to control animals (F = 16.34, P < 0.0001). The reduction was 30 % during nasal obstruction and 41 % at D90 in oral breathing males compared to control animals. Lung weights: A significant reduction of lung weight was observed only on D11 in the oral breathing group compared to the control group (F = 5.29, P = 0.003). The reduction was 14 % after three days of nasal obstruction. Adrenal gland weights: To determine if the absence of nasal respiration and the related transition to temporary forced oral breathing were associated with an enhanced level of stress, the weight of the adrenal glands was measured (Table 1). Animals exposed to nasal obstruction presented a greater adrenal gland specific weight compared to control animals (F=6.18, P=0.001). This significant difference was observed within 24 h after

the treatment (+20 %; t=2.79, P=0.008) and became more marked at D11 (+53 %; t=3.14, P=0.003). Nasal obstruction was thus associated with a significant increase of adrenal gland weight. This significant augmentation was still present at D90 (+21 %; t= 2.15, P=0.05). Testicle weights: There were no significant differences in testicular weights between oral breathing and control animals. The development of the testicles was not affected by nasal obstruction.

3.2. MHC isoform expression in neonatal (D9, D11) and adult (D90) rats

Based on densitometric analysis of the SDS-PAGE, the relative MHC isoform compositions of respiratory and orofacial muscles (LN, MS and AD) were determined. Results are shown in Fig. 2. and 3. The order of increasing electrophoretic mobility of developmental and adult MHC isoforms is as follows: emb, adult fast 2a, adult fast 2x, neo, adult fast 2b, and slow adult 1 type.

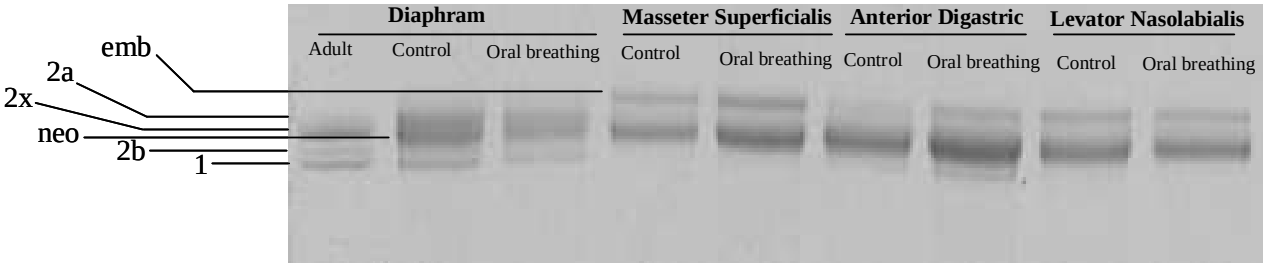


Fig. 2. Example of effects of temporary forced oral breathing on myosin heavy chain expression in four skeletal muscles: embryonic (emb), neonatal (neo), adult fast 2a, adult fast 2x, adult fast 2b, and slow adult 1 type.

In adult diaphragm, four MHC isoforms could be detected, in order of increasing electrophoretic mobility; the four fast types, MHC 2a, 2x, 2b, and the slow type, MHC 1 (Fig. 2 and 3.). These MHC isoforms were observed in both control and oral breathing animals in the diaphragm muscle. In orofacial muscles (LN, MS and AD), the three adult fast isoforms could be observed.

In the neonatal diaphragm muscle at D9 and D11, MHC neonatal and adult isoforms were observed in both control and oral breathing animals. In the orofacial muscles (LN, MS and AD) the relative expressions of embryonic and neonatal MHC isoforms were most abundant (> 85%).

In the diaphragm (Fig. 3.A) we found a significant difference in the relative distributions of MHC isoforms between control and oral breathing animals. Oral breathing was associated with an increase of MHC 1 in the diaphragm at D9 ($t = 3.24$, $P = 0.009$) and D11 ($t = 12.29$, $P < 0.0001$). At D90 (Fig. 3.A), we found a significant difference in the relative distribution of MHC isoforms between control and oral breathing animals. Oral breathing was associated with an increase in MHC1 of Diaphragm ($t = 3.83$, $P = 0.002$), 23.7% and 27.6% respectively in control and oral breathing rats.

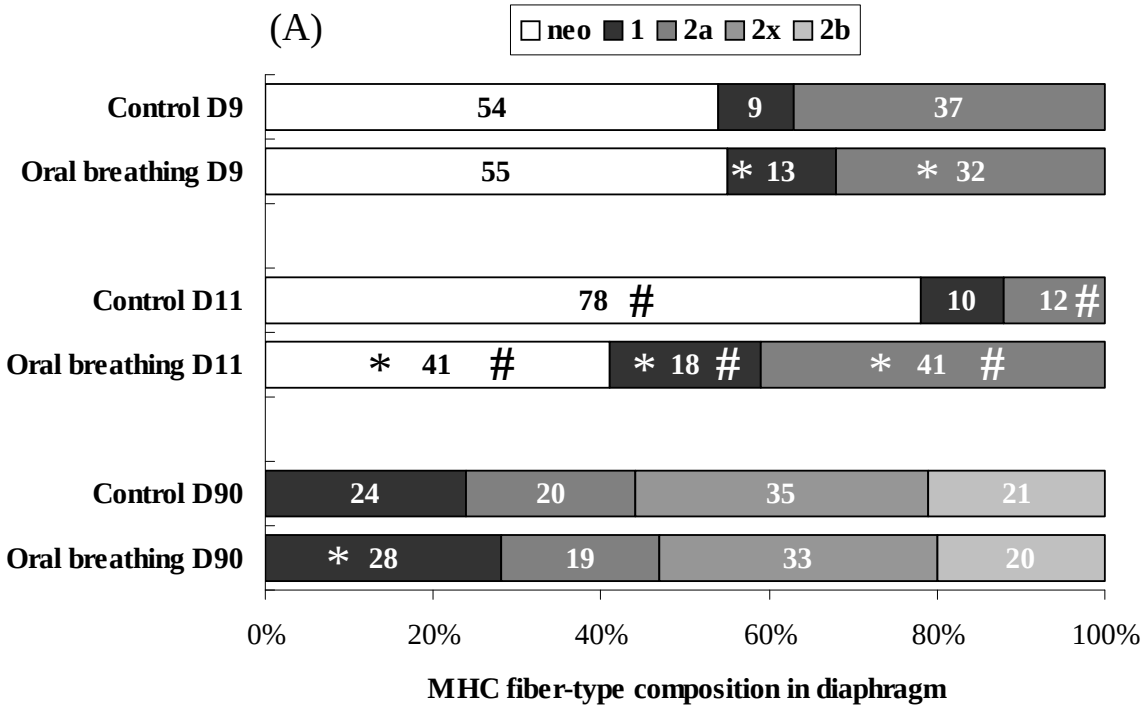


Fig. 3 A. Myosin heavy chain distribution in diaphragm muscle at 9, 11 and 90 days of age in control and rats exposed to temporary forced oral breathing. Values are percentages of total MHC (n = 7 per group), S.E.M values was for all groups included between 0.1 and 0.77. # significant difference from the previous day at $P < 0.05$. in young rats (D9-11). * Significant differences from control muscles at $t = -10.37$ to 26.03, $P < 0.03$ to < 0.0001.

In LN (Fig. 3.B), related to nasal breathing and rearing behaviour, we found no significant differences in the relative distribution of MHC isoforms between control and oral breathing animals at D9 and D11.

At D90, related to nasal breathing and rearing behaviour, oral breathing was associated with an increase in MHC 2a ($t = 26.03$, $P < 0.0001$) and a decrease in MHC 2x and 2b ($t = 8.20$, $P < 0.0001$ and $t = 19.33$, $P < 0.0001$, respectively).

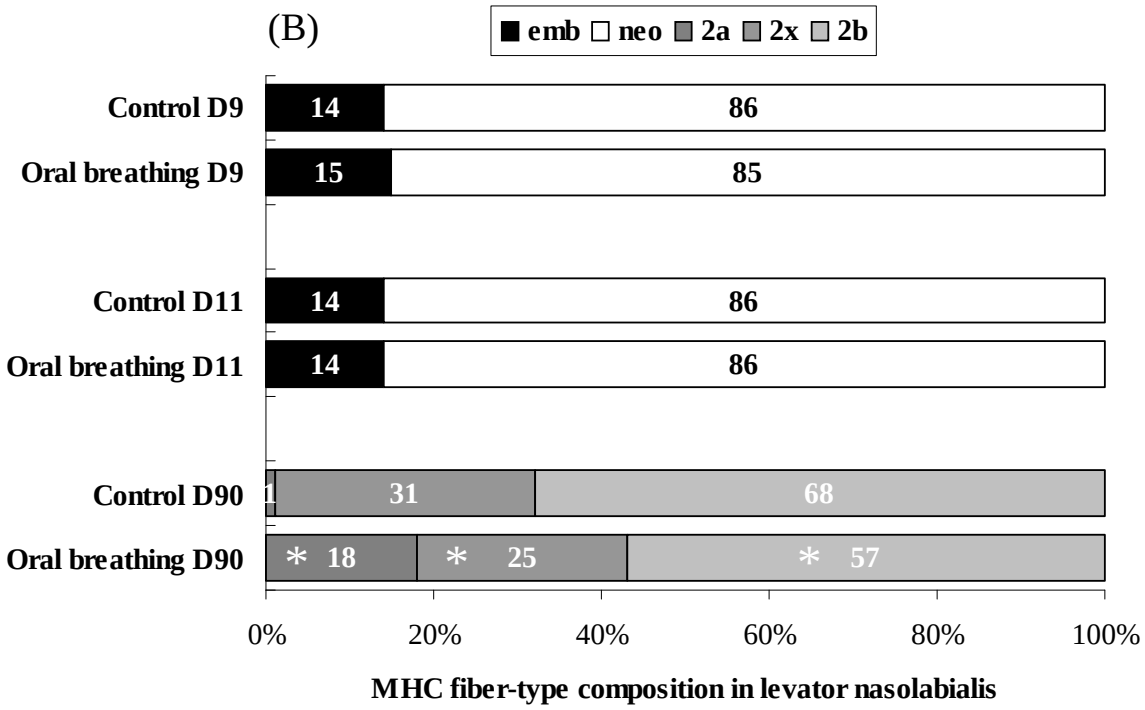


Fig. 3 B. Myosin heavy chain distribution in Levator nasolabialis muscles at 9, 11 and 90 days of age in control and rats exposed to temporary forced oral breathing. Values are percentages of total MHC (n = 7 per group), S.E.M values was for all groups included between 0.1 and 0.77. # significant difference from the previous day at $P < 0.05$. in young rats (D9-11). * Significant differences from control muscles at $t = -10.37$ to 26.03, $P < 0.03$ to < 0.0001.

In MS (Fig. 3.C), related to jaw lift, oral breathing were associated with a related decrease in embryonic MHC isoforms at D9 ($t = 2.22$ $P = 0.005$) and D11 ($t = 2.22$, $P = 0.005$).

At D90, related to jaw lift, oral breathing was related to an increase in MHC 2b ($t = 14.62$, $P < 0.0001$) to the detriment of MHC 2x ($t = 6.56$, $P < 0.0001$).

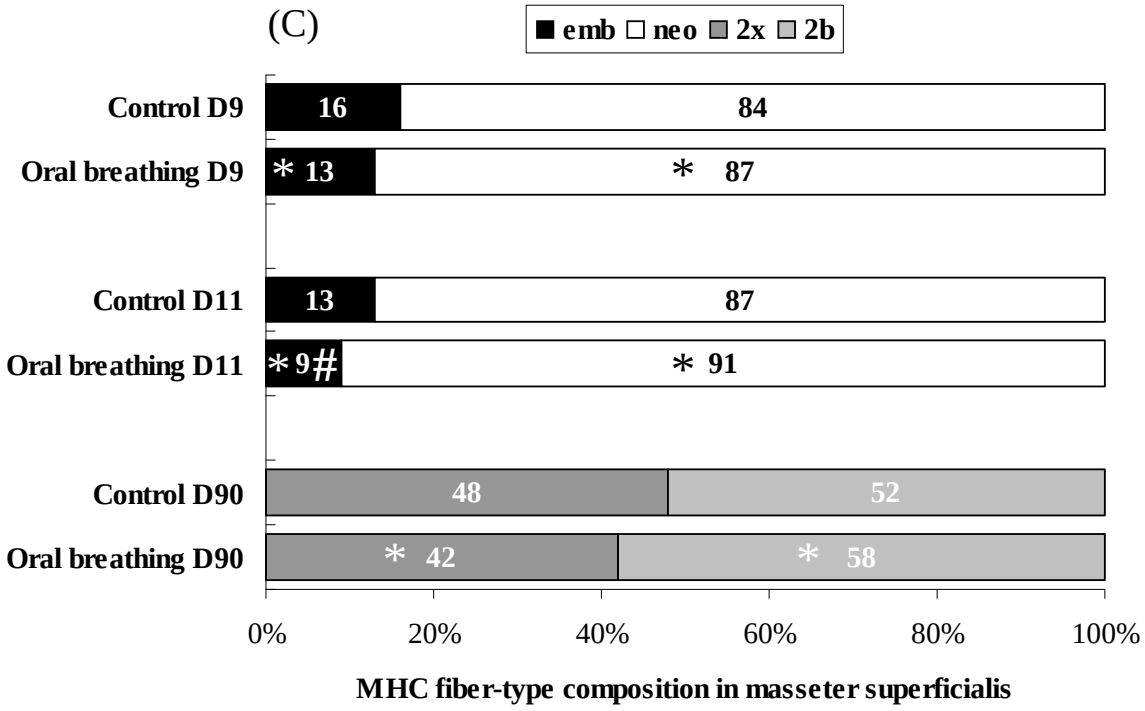


Fig. 3 C. Myosin heavy chain distribution in Masseter Superficialis muscles at 9, 11 and 90 days of age in control and rats exposed to temporary forced oral breathing. Values are percentages of total MHC (n = 7 per group), S.E.M values was for all groups included between 0.1 and 0.77. # significant difference from the previous day at $P < 0.05$. in young rats (D9-11). * Significant differences from control muscles at $t = -10.37$ to 26.03, $P < 0.03$ to < 0.0001.

In AD (Fig. 3.D), related to jaw depression, oral breathing was associated with decreased MHCneo at D9 ($t = 3.52$, $P = 0.005$) and D11 ($t = 10.37$ $P < 0.001$), with an increase of MHC1 at D9 ($t = 5.88$ $P = 0.002$) and D11 ($t = 5.85$, $P = 0.002$).

At D90, related to jaw depression, oral breathing was associated with an increase in MHC 2x ($t = 6.10$, $P < 0.0001$) and a decrease in MHC 2a ($t = 8.80$, $P < 0.0001$).

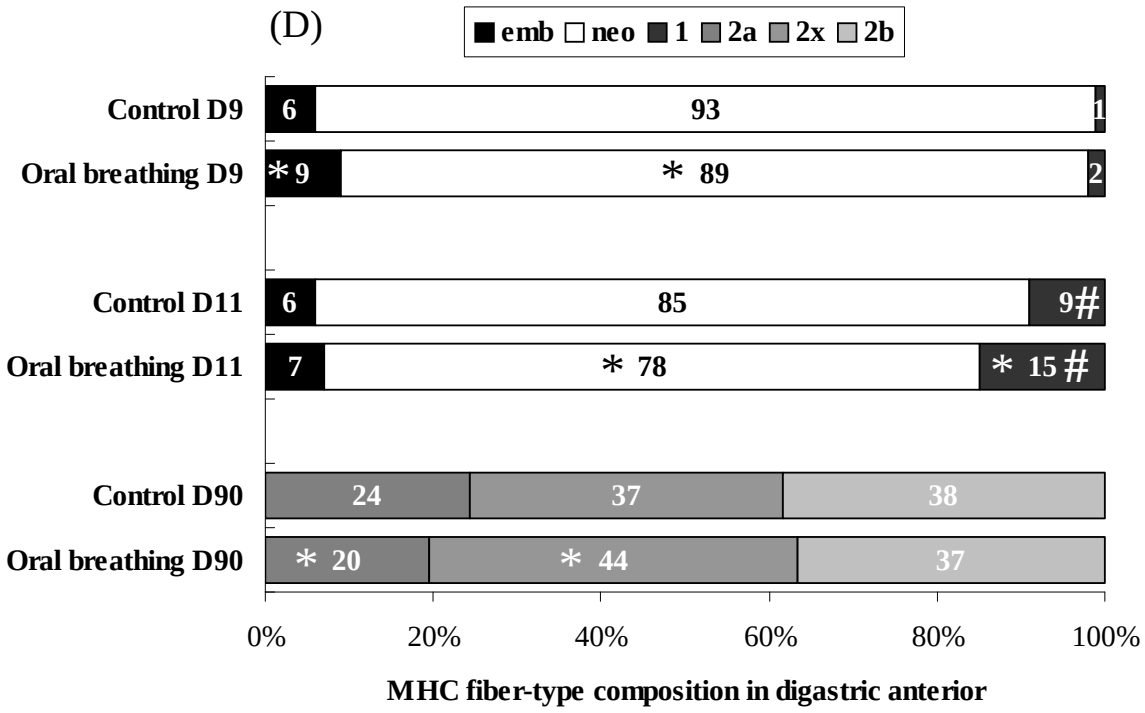


Fig. 3 D. Myosin heavy chain distribution in Anterior Digastric muscles at 9, 11 and 90 days of age in control and rats exposed to temporary forced oral breathing. Values are percentages of total MHC (n = 7 per group), S.E.M values was for all groups included between 0.1 and 0.77. # significant difference from the previous day at $P < 0.05$. in young rats (D9-11). * Significant differences from control muscles at $t = -10.37$ to 26.03, $P < 0.03$ to < 0.0001.

3.3. Hormone assays

As shown in Fig. 4.A, plasma corticosterone levels were significantly different between the experimental groups at D9 and D11. Twenty-four hours after treatment, nasal obstruction was associated with a significant augmentation in corticosterone ($F = 4.15$, $P < 0.0001$). At D11 plasma corticosterone levels were significantly increased in oral breathing rats ($F = 16.20$, $P < 0.0001$). At D90, plasma corticosterone level were no longer significantly different between the 4 days oral breathing and control animals ($P = 0.61$).

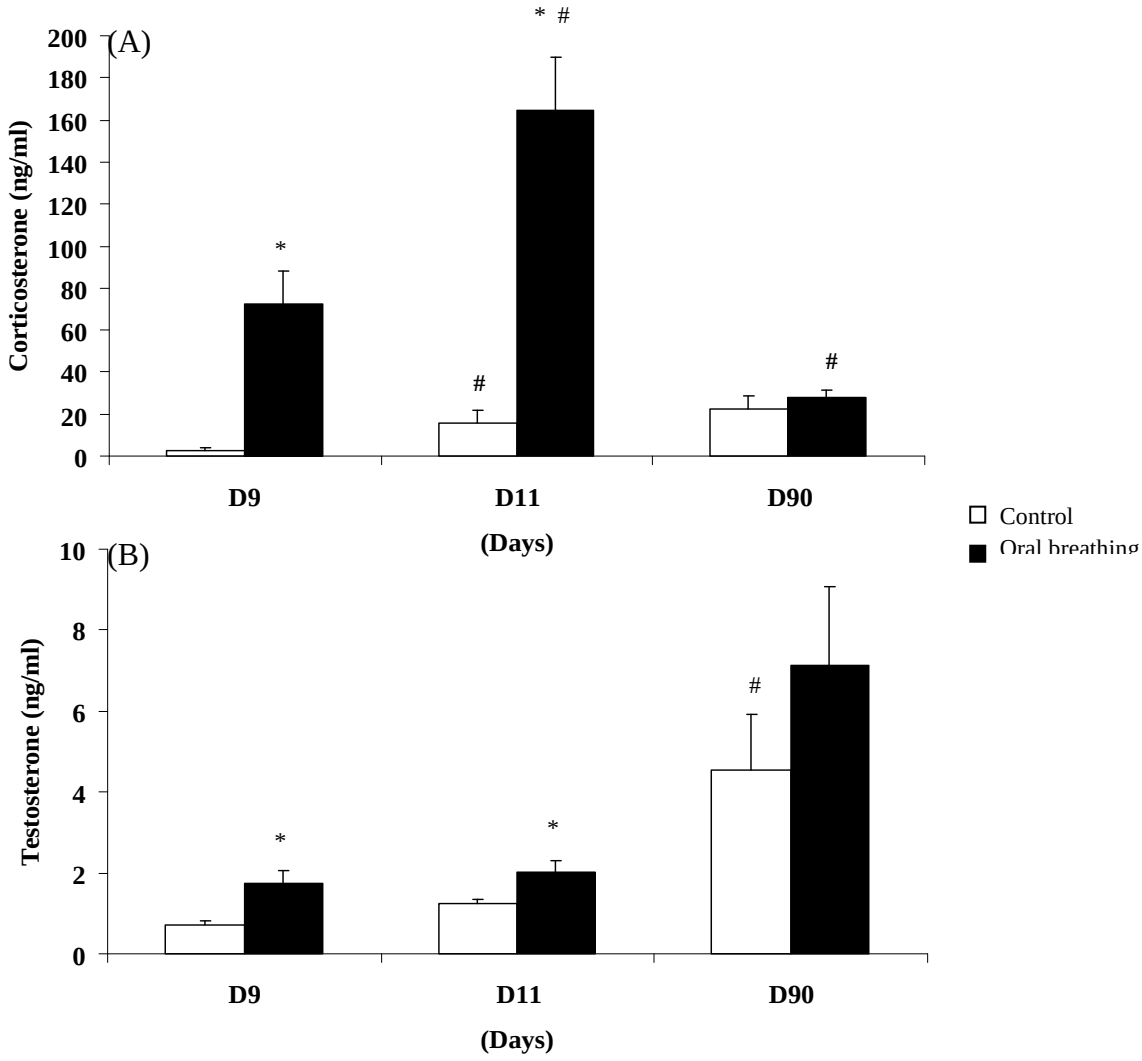


Fig. 4. Impact of early nasal obstruction on plasma corticosterone (**A**) and testosterone (**B**) levels at 9, 11 and 90 days of age in control and temporary forced oral breathing animals. Values are means ± S.E.M (n = 7 rats/group/age). Analysis of t-test: * significant difference from control group at 9 and 11 days at $P < 0.05$. # significant difference from the previous day at $P < 0.05$.

As shown in Fig. 4.B, plasma testosterone levels were significantly different between the experimental groups at D9 and D11. Twenty-four hours (D9) after treatment, nasal obstruction was associated with a significant augmentation of testosterone ($F = 15.20$, $P < 0.0001$). At D11, plasma testosterone levels were significantly increased in oral breathing rats (($F = 12.15$, $P < 0.0001$). At D90, plasma testosterone levels were significantly higher in both the oral breathing group and the control group compared to D11.

Figure 5.A shows that 24 h after the treatment (D9), thyroxin (T4) concentration was significantly reduced in nasal obstruction compared to control animals (F=11.92, P < 0.0001): At D9, plasma T4 levels were significantly reduced by nasal obstruction (-24%; t = 3.69; P=0.0009). This difference in plasma T4 levels was maintained at D11 (-15 %; t = 4.20, P = 0.0002), and at D90 (-12%, t = 3.05, P = 0.04).

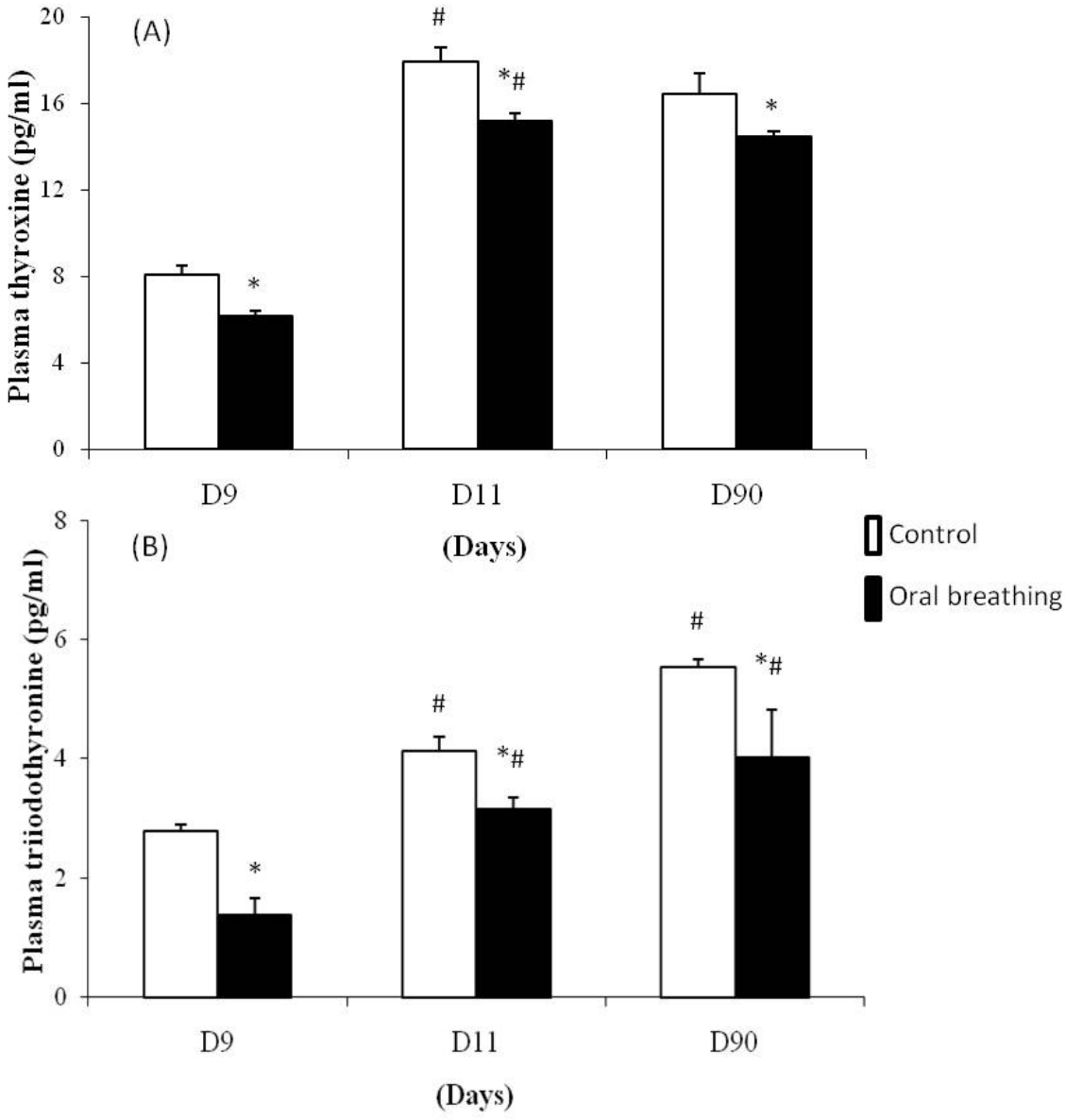


Fig. 5. Impact of early nasal obstruction on plasma thyroxine (**A**) and triiodothyroxine (**B**) levels at 9, 11 and 90 days of age in control and temporary forced oral breathing animals. Values are means ± S.E.M (n = 7 rats/group/age). Analysis of t-test: * significant difference from control group at 9, 11 and 90 days at P < 0.05. # significant difference from the previous day at P < 0.05.

Figure 5.B shows that plasma triiodothyronin (T3) levels were significantly different between the experimental groups at all ages tested ($F = 14.51$, $P < 0.0001$). Indeed animals with nasal obstruction had lower levels of T3 throughout the period studied. The reduction was 49 % at D9, 23 % at D11 and 27 % at D90.

4. Discussion

These results have shown that in animals with short term nasal obstruction-induced oral breathing there are increases in MHC neonatal and adult type 1 isoforms in two muscles involved with oral breathing, *Masseter Superficialis* (MS) and *Anterior Digastric* (AD). During this oral breathing period no changes were observed in the *Levator Nasolabialis* (LN) muscle involved with nasal breathing. Reversible nasal obstruction was associated with reduced growth of the rat pups during oral breathing, decreased growth of the olfactory bulbs lasting into adulthood, and an initial decrease in lung growth which had recovered at 90 days. After only 1 day of nasal obstruction adrenal hypertrophy was observed and this lasted into adulthood. The consequent plasma levels of “stress” hormones were increased during the obstruction but normal by adulthood. An increase in plasma testosterone was observed during the obstruction (but not in adulthood), and a decrease in thyroid hormone levels observed throughout. Thus we have shown clearly that very short term nasal obstruction, *i.e* oral breathing, leads to long term respiratory muscle adaptation and significant hormonal changes.

Our results show that nasal obstruction causes early changes in structural development of the respiratory muscles, which begins within 24h after obstruction and is maintained at least until adulthood. Indeed, in oral breathing animals we have shown an acceleration of structural development of the respiratory muscles during the period of nasal obstruction. The period of nasal obstruction was associated with modifications in MHC isoform expression. During the nasal obstruction period, our results showed a decrease in MHCneo (the predominant neonatal isoform) to the benefit of MHC 1, 2a (the mature isoform) in the diaphragm. In addition, in oral breathing animals the muscles related to jaw movement presented a relative increase in expression of MHCneo to the detriment of MHCemb (the embryonic isoform) in MS, and an increase in MHC 1 (the mature isoform) to the benefit of MHCemb and MHCneo isoforms in AD. These results showed that nasal obstruction induced accelerated structural development of the breathing muscles. During development, muscles usually change

directly from embryonic to neonatal to fast, or from embryonic to neonatal to slow isoforms (d'Albis *et al.*, 1989). Geiger *et al*., (2006) have shown that MHCneo increases between D0 to D14 and there after decreases to disappear at the age of 28 days. Our results showed that between D9 and D11 MHCneo increased in control and decreased in oral breathing animals, which is in accordance with the results of Geiger *et al*., (2006) for control animals. This leads apparently to an accelerated maturation for the forced oral breathing animals because the decrease of MHCneo was to the benefit of MHC adults isoforms. In the adult a similar profile has been found. Indeed, in male rats aged 90 days we observed an increase in the MHC 1 isoform in the diaphragm. At adulthood the LN showed an increase in the 2a isoform at the expense of 2x and 2b isoforms. MS and AD muscles showed antagonist profiles with a decrease in the MHC 2x isoform in MS and an increase in AD muscles.

Thus, oral breathing rats presented a profile in MHC adapted to the transition from nasal to oral breathing, in other words a change facilitating respiration. This is in agreement with results in the literature (Martrette *et al*., 1998) that show that environmental conditions (such as hypergravity) could induce structural changes in the development of the muscles.

MHC isoform expression may have a profound effect on muscle fibre contractile and energetic properties (Sieck & Regnier, 2001 ; Watchko & Sieck, 1993). Indeed, fibres expressing MHC 1 generate less maximum specific force, slower shortening velocity and greater resistance to fatigue than fibres expressing fast MHC isoforms. Among fast fibres, those expressing MHC 2x and 2b generate greater maximum specific force, faster shortening velocity and lower resistance to fatigue than fibers expressing MHC 2a. In addition, in oral breathing animals, the muscles related to mouth opening, AD, will be more susceptible to resist fatigue than MS. This result could be explained by a different control of muscle activity between MS and AD. Indeed, van Wessel *et al.,* (2005) have recently shown that MS and AD presented differences in electromyographic (EMG) activity (in terms of bursts number) during daily activity in the rabbit. In contrast to AD, MS showed a bimodal burst distribution. The authors interpreted this result as the consequence of an additional postural activity for MS only, consisting of many short low-amplitude bursts. In addition, temporary forced oral breathing could produce some behavioural modifications in both nursing and breathing behaviours (mouth opening and rearing behaviour) associated with alterations in specific electromyographic activity of respiratory muscles. Thus, behaviour and related

electromyographic activity are probably not the only factors acting upon MHC distribution.

Furthermore, according to these observations, the present investigation has shown that in the long term diaphragm and LN become more resistant to fatigue following temporary forced nasal obstruction. The changes in profile of the MHC in the LN of oral breathing rats could be explained by a decreased solicitation, in fact flaring appears modified in these rats, and they are less able to recognise and respond to receptive females (unpublished results).

Our study has shown that nasal obstruction caused early structural development changes of the respiratory muscles, starting within twenty-four hour of obstruction and remaining throughout the long term. Gelhaye *et al.,* (2006a) showed that early nasal obstruction caused structural modification of respiratory muscles at D21, and now we have shown that these changes start very early during the period of nasal obstruction (D9-D11) and that they are maintained. Rodent studies have shown the influence of testosterone on the expression and maintenance of MHC fibres especially type IIb (Prezant *et al.,* 1997 ; Eason *et al.,* 2000). This would help to explain the increase in type IIb fibres in the MS muscle. However, the different muscles appeared to react differently to the increased plasma testosterone levels, with reductions in type IIb seen in LN and DA and no change seen in the diaphragm. Further, more detailed, analysis of the effect of testosterone on muscle MHC fibre type expression appears necessary.

Our results showed that animals with nasal obstruction presented an impaired olfactory bulb development during the period of nasal obstruction (D9, D11) as well as in the long-term (D90). Several studies have shown the impact of early nasal obstruction in rats. However, no study has shown that its impact is rapid and lasts through the long term. Gelhaye et al., (2006a) have shown the impact of early nasal obstruction (D8) on the development of the olfactory bulbs at weaning (D21). Loranca & Salas (2001) have also shown that early nasal obstruction (D3) causes atrophy of the olfactory bulbs for up to 70 days. Our results show that atrophy of the olfactory bulbs begins within 24 hours after nasal obstruction (D9) and remains for the long term (D90). Furthermore, spontaneous reopening of the nasal passage is not complete therefore the olfactory bulb may not receive sufficient stimulation for normal responsiveness, as already mention above with respect to flaring.

Nasal obstruction is associated with the establishment of oral breathing, which causes short-term stress and disruption in development of the male rat. Indeed, our

results have shown atrophy of the lungs during the period of nasal obstruction. To our knowledge, no studies have shown an impact of nasal obstruction on lung development. Nasal obstruction and the associated switch to temporary forced oral breathing were correlated with adrenal hypertrophy (72 hours after treatment) and increased corticosterone plasma levels (24 hours after treatment), but that this result was not maintained in the long term (D90). It is well known that there is a direct relation between stress exposure and increased adrenal gland weight (Basset & West, 1997). Accordingly, we suggest that nasal obstruction and its consequences *ie* low of body weight (perhaps *via* nutritional depletion), possible hypoxia, as well as olfactory deprivation related to social deprivation represent a multi-factorial stressful situation, which enhances global activity of the hypothalamo-pituitary-adrenal axis. This result could be correlated directly with atrophy of the olfactory bulbs. Even if the mechanism is still poorly understood today, several studies have shown links between the olfactory systems and gonadotropin. Pieper *et al.* (1990) showed that bulbectomy in hamsters resulted in an increase in serum gonadotropin approximately one-half of the increase seen after castration. This suggests that the olfactory bulb has an influence on gonadotropin secretion which might be mediated by altering gonadal steroid feedback. Neurons throughout the olfactory and vomeronasal pathways in the limbic system have receptors for androgens and estrogens. In rats, olfactory bulb removal has been reported to decrease androgen receptor binding in both the amygdala and the hypothalamus (Lumia *et al.*, 1987). If a similar reduction occurred in hamsters, this could explain why there was a decrease in the responsiveness to testosterone feedback on gonadotropin secretion.

Animals exposed to nasal obstruction showed a decrease in plasma T4 and T3 levels up to 90 days after the nostril reopening. Different factors could be involved in these modifications among which are nutritional depletion and the associated secretion of glucocorticoids. A suppressive impact of nutritional deprivation on T3 and T4 levels has been shown (Kasdallah *et al.*, 2005) and these effects could be mediated by activation of the hypothalamo-pituitary-adrenal axis (Benker *et al.*, 1990). Hypothyroidism observed in animals exposed to nasal obstruction could produce several deleterious effects such as a maturation defect of the central nervous system (Koibuchi & Iwasaki, 2006) or a decrease of basic metabolism and thermogenesis (Silva, 2003).

In conclusion, the present study has shown that the structure of respiratory muscles and the levels of plasma hormones can be altered by temporary forced oral breathing. The observed changes began very early during the period of nasal obstruction and were maintained over the long term. Indeed, in animals exposed to temporary forced oral breathing, muscles involved in respiratory activity presented an increased relative expression of fatigable MHC isoforms. These modifications could contribute in some part to various human pathologies, and it would be interesting to specify the factors that act to produce these changes in muscular structures and what other morphological changes could be involved. An analysis of histochemical profile of MHC was necessary to determine if a switch of MHC fibre type during the early period of oral breathing was in part at the origin of the MHC profile observed in oral breathing adult rats. It will also be necessary subsequently to measure the degree of hypoxia and hydration in pups exposed to temporary forced oral breathing and to study the relation between chronic nasal obstruction, nutrition and craniofacial growth. Furthermore, nasal obstruction is considered a risk factor in sleep-disordered breathing (Rombaux *et al.*, 2005 ; Armengot *et al.*, 2008 ; Craig *et al.*, 2008) which in children and adults has a very negative impact on quality of life with increased daytime sleepiness (Udaka *et al.*, 2006). This symptom resembles that of obstructive sleep apnea caused by episodes of upper airway obstruction leading to episodic hypercapnic hypoxia which alters upper airway muscle structure and fibre type expression in ways somewhat similar to those reported here (McGuire *et al.*, 2002). This could indicate that our model of temporary nasal obstruction could be an appropriate model for looking at potential changes in hormones and other physiological parameters of rhinitis or other temporary obstructive nasal breathing pathology.

Article 4

Craniofacial development and physiological state after early oral breathing in rat

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European J Oral Science (soumis)

1 - Introduction

The influence of respiratory function in development of orofacial structures has been widely discussed. According to MOSS's Theory of Functional Matrices (Moss & Salentijn 1969), nasal breathing allows proper growth and development of the craniofacial complex interacting with other functions such as mastication and swallowing (Prates et al., 1997). This theory is based on the principle that facial growth is closely related to functional activity represented by different components of the head and neck region. Craniofacial growth is related to various factors, such as hormones (Hwang & Cha, 2004 ; Peltomäki, 2007), heredity factors and mechanical stress (Bresin et al., 1999 ; Delatte et al., 2004 ; Oshikawa et al., 2004). Chronic nasal obstruction is a non-specific condition observed in many pathological conditions, *e.g.* allergic rhinitis, rhinosinusitis, adenoid hypertrophy and nasal polyps. These conditions are associated with ogival palate and excessive development of the vertical axis of the facial skeleton (dolicocephalia or "long face syndrome") (Subtelny, 1975; Smith & Gonzalez, 1989).

Nevertheless, because this disorder is not life threatening (at least in adults) its importance could be underestimated. Impaired nasal breathing results in obligatory oral breathing, which can to be divided into two components: chronic absence of active nasal respiration that results in an olfactory deprivation (Jennings et al. 1985) and chronic mouth opening (Meisami, 1976).

If we consider the doctrine of functional matrices, nasal obstruction associated with oral breathing may have an impact on growth orientation of the facial skeleton

structure (Schlenker et al., 2000). Children with chronic oral breathing, whether due to nasal obstruction or not, develop several morphological disorders during growth resulting in unfavorable dental-facial complex development (Schendel et al. 1976 ; Hulcrantz et al., 1991 ; Harari et al., 2010). Chronic oral breathing is also known to be a contributing factor in deviant facial growth patterns in pre-school children (Yang et al., 2002 ; Mattar et al., 2004).

Nasal obstruction, whatever the origin, induces oral breathing which may be considered as a stressful situation. Stressful situations correspond to particular changes in environmental conditions that induce modifications in different physiological parameters such as plasma hormonal levels. For example, stressful situations produce an adrenal hypertrophy and an increase of plasma glucocorticoid levels (Gomez et al., 1996; Padzys et al., 2011a) which are known to induce alterations in craniofacial development (Arcus & Kagan, 1995 ; Fujita et al., 2008). Plasma levels of thyroid hormones can be reduced in stressful situations and these hormones are very important in the normal development of vertebrate skeletal ossification (Shao et al., 2006). Thyroid hormones are required for skeletal development and establishment of peak bone mass. Hypothyroidism in children results in growth retardation with delayed skeletal development, whereas thyrotoxicosis accelerates bone maturation. In adults, T3 regulates bone turnover and bone mineral density, and normal euthyroid status is essential to maintain optimal bone strength (Williams, 2009).

Scarano *et al.* (1998) showed that chronic nasal obstruction for 3 months performed in rats aged 28 days lead to stunted growth and development of the nasomaxillary complex. Different mechanisms may be at the origin of these changes; eg functional stimulation was associated with mouth breathing. However, other mechanisms are possible.

No study has shown the impact in the short and long term of early nasal obstruction associated with chronic oral breathing on craniofacial development in the rat. The aim of the present investigation was to evaluate the effect of early short term (4 days) nasal obstruction associated with chronic oral breathing, on craniofacial growth, and its the long term (D90, adult) impact. Thus, our hypothesis was that oral breathing would have a significant effect of craniofacial development during the very short period

of forced oral breathing. The effect of early oral breathing on various organ weights (olfactory bulbs, lungs) and on hormonal status was studied also. In particular the stress response and plasma levels of thyroid hormones (T3 and T4) were evaluated to determine if these hormones could be implicated in craniofacial development during early oral breathing.

2 - Methods

2.1. Animal care

Male and female Wistar rats (iops IFFA- CREDO) were used in these experiments. These pups were born in the laboratory from 14 litters (total number 84), culled to 7 pups per litter to ensure normal body growth. The animals were housed in standard cages under controlled temperature conditions (22 ± 1°C). Food and water were available *ad libitum* throughout the experiment. From birth, the rats were kept on a reversed 12:12 light-dark cycle (dark period 08:00-20:00h).

2.2. Nasal obstruction procedure

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (no. 85-23, revised 1996), the recommendations of the European Community Council for the Ethical Treatment of Animals (no. 86/609/EEC) and the regulations of the University of Nancy 1. All efforts were made to minimize animal suffering.

At the post natal age of 8 days (D8), the litters were first anesthetized; all animals were weighed and then randomly divided into one control group and one experimental group (oral breathing). Bilateral nasal obstruction resulting in forced oral breathing was performed in the experimental animals (at least 7 per age and per sex) as described previously (Gelhaye et al., 2006a,b). The selected method consisted in the cauterization of the external nostrils, which is the most common and simple procedure allowing reversible nasal obstruction in neonates. The tissue surrounding the external nostrils was burned by placing a surgical cauterizing instrument (1 mm in diameter) on the nostrils, consequently occluding the orifice of the nostrils without mechanical or chemical

damage to the olfactory mucosa. This procedure induced complete nasal obstruction between D8 and D11 with 100 % of the nostrils reopened at D15.

In the control group, the nostrils were not sealed but the cauterizing instrument was placed about 1-2 mm above each nostril (at least 7 pups per age and per sex). After cauterization, the nostrils were washed with chlortetracycline (Aureomycine Evans 3%) to prevent infection. Control and oral breathing animals were kept warm (37°C) for 30 min and then returned to their mothers. The sampling experiments were conducted during complete nasal obstruction day 9 (D9) and day 11 (D11) and at 90 days after post-reopening of the nostrils, ie at the beginning of adulthood. In order to avoid interference between the different litters, one male and one female from each litter and at each age were randomly used to experiments. For this we needed 84 subjects from 14 litters.

2.3. Sample collection

At D9, D11 or D90, seven rats per group (control and oral breathing) per age and per sex were removed, immediately humanely killed, weighed and intracardiac blood samplings (500 – 1000 µl) were performed between 11h and 12h for corticosterone, thyroxine and triiodothyronine measurements. Blood was collected within 1-2 min using sterile heparinised syringes fitted with a 26-G needle. Plasma was immediately separated by centrifugation at 4°C (15 min at 3000 rpm) then the extracts were aliquoted and stored at -18°C until the time of assay (1-2 days).

After blood sampling, cephalometric analysis was realized, and then olfactory bulbs and lungs were removed bilaterally and weighed.

2.4. Cephalometric analysis

Teleradiographs in both submento-vertex and latero-lateral projections were carried out by means of a craniostat as described by Scarano *et al.* (1998). Cephalometric measurements were taken with an integration scanner Bio-Rad GS-800 and analyzed with the program Quantity One 4.2.1., the precision of measure was one hundredth of a millimetre.

Cephalometric analysis was carried out considering the following points:

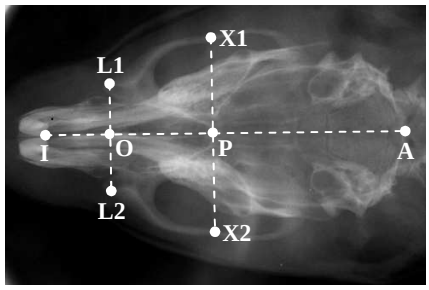

Figure 1: Submento-vertical projection

Figure 1 represents a teleradiograph in the submento-vertex projection. (I), superior interincisive point; (A), most posterior point of the occipital bone; (A-I line), through points A and I, indicating the overall length of the skull; (P), basis of the sphenoid bone; (L1), most anterior and superior point in the malar process of the right maxilla; (L2), most anterior and superior point in the malar process of the left maxilla; (O), intersection point of the premaxillary-palatal junction on the A-I line; (X1), intersection point between a straight line passing through point P and normal to the median plane, and the internal border of the right zygomatic arch; (X2), intersection point between a straight line passing through point P and normal to the median plane, and the internal border of the left zygomatic arch; (X1-X2 line), transversal development of the most posterior region of the fossa cranica media; (L1-O line), medio-lateral development of their right maxilla; (L2-O line), medio-lateral development of ther left maxilla; (L1-L2 line), overall development of both maxillae.

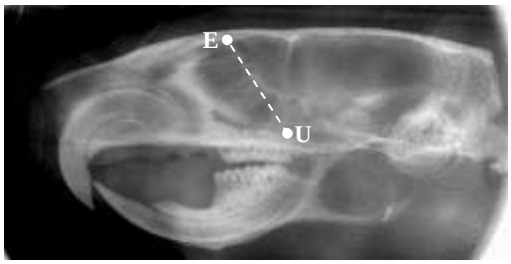

Figure 2 : Latero – lateral projection

Figure 2 represents a teleradiograph in the Latero – lateral projection. (E), intersection between the frontal bone and the most superior and anterior point of the ethmoid; (U), intersection between the maxillary sinus and the distal surface of the third superior molar tooth; (E-U line), overall height of the nasomaxillary complex (Fig. 2).

2.5. Hormone assays

Corticosterone concentration was measured without an extraction procedure, using a commercially available EIA kit and performed according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentrations of corticosterone in plasma samples were calculated from a standard curve and expressed as ng/ml.

Thyroxine (T4) and **triiodothyronine** (T3) were assayed using commercial RIA kits and performed according to the manufacturer's guidelines (Immunotech SA, Marseille, France). The concentrations of T4 and T3 in plasma samples were calculated from standard curves and expressed as pg/ml. The intra- and inter-assay coefficients of variation were respectively under 6.7 and 6.5 % for T4 and under 6.4 and 5.5 % for T3.

2.6. Statistical analysis

The results were expressed as group means ± SEM. Student's t-test was used to establish the comparison between control and oral breathing animals since all data were normally distributed. Group differences were determined using ANOVA. Analysis of Specific mean comparisons were then made using PLSD Fischer test. Differences were considered significant at $P < 0.05$.

3 - Results

3.1. Cephalometric analysis

Cephalometric studies showed that in males (table 1), oral breathing was associated with a decrease in nasomaxillary height at the three ages ($F = 14.47$, $P < 0.0001$).

In the female group (table 2) oral breathing was associated with a decrease of the nasomaxillary complex in the short term ($F = 7.45$, $P = 0.002$). No differences were observed in adult females ($F = 1.51$, $P = 0.92$).

Oral breathing was associated with a delay in longitudinal skulbase growth in both sexes (tables 1 and 2). Indeed oral breathing resulted in a delay in development of the longitudinal skulbase in males ($F = 17.95$, $P < 0.0001$) and females ($F = 15.18$, $P < 0.0001$).

Males	9 days	11 Days	90 Days
Control group			
Height (U-E)	8.25 ± 0.07	9.20 ± 0.16	14.35 ± 0.02
Length (A-I)	22.92 ± 0.02	24.96 ± 0.02	42.40 ± 0.01
Width :			
L1-O	2.54 ± 0.16	3.6 ± 0.17	5.33 ± 0.02
L2-O	2.47 ± 0.13	2.82 ± 0.01	5.42 ± 0.01
L1-L2	5.01 ± 0.29	6.08 ± 0.28	10.75 ± 0.03
X1-X2	11.34 ± 0.03	13.29 ± 0.07	14.90 ± 0.02
X1-P	5.64 ± 0.01	6.53 ± 0.06	7.58 ± 0.01
X2-P	5.70 ± 0.02	6.36 ± 0.01	7.32 ± 0.01
Oral breathing			
Height (U-E)	7.01 ± 0.01*	8.01 ± 0.02*	13.25 ± 0.02*
Length (A-I)	21.31 ± 0.41*	23.11 ± 0.03*	40.71 ± 0.02*
Width :			
L1-O	2.84 ± 0.15	3.32 ± 0.12	5.33± 0.01
L2-O	2.65 ± 0.10	2.80± 0.03	5.41 ± 0.10
L1-L2	5.49 ± 0.16	6.12 ± 0.02	10.74 ± 0.11
X1-X2	11.20 ± 0.05	13.20 ± 0.02	14.62 ± 0.11
X1-P	5.61 ± 0.02	6.55 ± 0.01	7.42 ± 0.01
X2-P	5.59 ± 0.03	6.33 ± 0.01	7,20 ± 0.01

Table 1. Effects of chronic oral breathing on craniofacial growth at ages 9, 11, and 90 days in male controls and animals exposed to nasal obstruction. Values are means ± S.E.M. (n = 7/group/age). ANOVA summary: * significantly different from control group at $P < 0.05$.

Considering the anterior development of the right (L1-O) and left (L2-O) maxilla no significant differences were found at the three ages in males (L1-O: $F = 1.58$, $P = 0.22$; L2-O: $F = 0.54$ $P = 0.64$) and females (L1-o: $F = 0.78$, $P = 0.24$; L2-o: $F = 2.16$, $P = 0.12$).

Females	9 days	11 Days	90 Days
Control group			
Height (U-E)	8.00 ± 0.02	9.20 ± 0.01	13.08 ± 0.02
Length (A-I)	22.70 ± 0.03	23.86 ± 0.02	40.12 ± 0.01
Width :			
L1-O	2.10 ± 0.04	2.33 ± 0.01	4.45 ± 0.01
L2-O	2.18 ± 0,01	2.36 ± 0.01	4.80 ± 0.01
L1-L2	4.28 ± 0.05	4.69 ± 0.02	9.25 ± 0.02
X1-X2	10.77 ± 0.02	12.72 ± 0.02	14.20 ± 0.03
X1-P	5.24 ± 0.02	6.34 ± 0.01	6.41 ± 0.01
X2-P	5.43 ± 0.01	6.14 ± 0.01	6.40 ± 0.02
Oral breathing			
Height (U-E)	6.98 ± 0.02*	7.56 ± 0.02*	13.04 ± 0.02
Length (A-I)	21.15 ± 0.01*	22.2 ± 0.01*	38.05 ± 0.02*
Width :			
L1-O	2.21 ± 0.08	2.36 ± 0.02	4.46 ± 0.01
L2-O	2.13 ± 0.02	2.35 ± 0.01	4.78 ± 0.01
L1-L2	4.34 ± 0.02	4.74 ± 0.02	8.08 ± 0.02
X1-X2	10.95 ± 0.03	12.40 ± 0.02	14.22 ± 0.03
X1-P	5.23 ± 0.02	6.35 ± 0.01	6.43 ± 0.02
X2-P	5.72 ± 0.01	6.15 ± 0.01	6.42 ± 0.01

Table 2. Effects of chronic oral breathing on craniofacial growth at ages 9, 11, and 90 days in female controls and animals exposed to nasal obstruction. Values are means ± S.E.M. (n = 7/group/age). ANOVA summary: * significantly different from control group at $P < 0.05$.

This trend may also explain the lack of difference of the total anterior transverse diameter (L1-L2) between control and oral breathing males and females (tables 1 and 2). Oral breathing appeared to have no role in the growth of the posterior transverse diameter (X1- X2) in males ($F = 0.85$, $P = 0.72$) and females ($F = 0.44$, $P = 0.31$).

3.2. Morphometric characteristics

Before the treatment, at 8 days of age, the weights of control and oral breathing pups were not significantly different: 17.8 ± 0.5 g for the males and 17.6 ± 0.2 g for the females ($F = 3.04$, $P = 0.11$).

Table 3 shows that in males there was a significant difference ($F = 4.17$, $P = 0.002$) in body weight at D9 and D11 between control and oral breathing rats. No

differences were observed at adulthood (D90) between control and oral breathing male ($F = 2.52$, $P = 0.82$). To check whether nasal obstruction affected the development of olfactory bulbs, their mass was measured. A significant specific reduction in olfactory bulb mass was found for the three ages in the oral breathing group compared to control animals ($F = 16.34$, $P < 0.001$). The reduction percentage was around 30 % during nasal obstruction (D9-D11) and of 41 % at D90 in oral breathing males compared to control animals.

Males	9 days	11 Days	90 Days
Control group			
Body weight (g)	18.60 ± 0.49	23.08 ± 0.59	408.47 ± 9.6
Bulbes olfactifs (mg/g)	1.62 ± 0.13	1.21 ± 0.03	0.22 ± 0.01
Oral breathing			
Body weight (g)	15.51 ± 0.48*	19.75 ± 0.79*	394.18 ± 8.65
Bulbes olfactifs (mg/g)	1.12 ± 0.12*	0.88 ± 0.04*	0.13 ± 0.01*

Table 3. Effects of chronic oral breathing on body weight (g) and olfactory bulbs specific weight (mg/g) at ages 9, 11, and 90 days in male controls and animals exposed to nasal obstruction. Values are means ± S.E.M. (n = 7/group/age). ANOVA summary: * significantly different from control group at $P < 0.05$.

In females (table 4) no difference was observed one day after treatment ($F = 2.08$, $P = 0.63$), but there was a significant decrease in body weight at D11 in the oral breathing group ($F = 5.50$, $P = 0.001$). No differences were observed at adulthood (D90) between control and oral breathing female rats ($F = 2.62$, $P = 0.28$).

Females	9 days	11 Days	90 Days
Control group			
Body weight (g)	17.02 ± 0.10	20.87 ± 2.30	287.27 ± 5.50
Bulbes olfactifs (mg/g)	1.56 ± 0.10	1.22 ± 0.04	0.35 ± 0.01
Oral breathing			
Body weight (g)	17.26 ± 2.10	16.12 ± 1.10*	247.42 ± 6.80
Bulbes olfactifs (mg/g)	1.45 ± 0.16	0.86 ± 0.09	0.23 ± 0.01*

Table 4. Effects of chronic oral breathing on body weight (g) and olfactory bulbs specific weight (mg/g) at ages 9, 11, and 90 days in female controls and animals exposed to nasal obstruction. Values are means ± S.E.M. (n = 7/group/age). ANOVA summary: * significantly different from control group at $P < 0.05$.

In the oral breathing female group, table 4 shows a specific reduction in olfactory bulb weight two days after treatment (D11) and at D90 ($F = 29.31$, $P < 0.001$).The reduction percentage was 30 % at D11 and 34 % at D90 in oral breathing compared to control females.

Lung weights were measured to check whether nasal obstruction affected their development. In the both sexes oral breathing was associated with a significant specific reduction in lung weight at D11 in male (18.83 ± 0.54 mg/g vs 16.20 ± 0.02 mg/g; $F = 5.29$, $P = 0.003$) and female (20.47 ± 0.10 mg/g vs 17.01 ± 0.58 mg/g; $F = 20.35$, $P < 0.001$) rats. The delay of lung weight growth was 14 % in male and 17 % in female oral breathing compared to control animals. No differences were observed in males and females at adulthood ($F = 3.58$, $P = 0.38$).

3.3. Hormone assays

As shown in table 5, plasma corticosterone levels were significantly different between the experimental groups at D9 and D11. One day after treatment, nasal obstruction was associated with a very significant augmentation in basal plasma corticosterone levels in males at D9 and D11 ($F = 16.20$, $P < 0.0001$). No difference was observed in adult males ($F = 11.20$, $P = 0.61$).

Table 5 shows that plasma triiodothyronine levels were significantly different between the experimental groups in males at all ages tested ($F = 14.51$, $P < 0.0001$). Indeed animals with nasal obstruction had lower levels of triiodothyronine throughout the period studied. The reduction was 49 % at D9, 23 % at D11 and 27 % at D90. Table 5 shows also that in males oral breathing was associated with a decrease in tyroxine concentration ($F = 11.92$, $P < 0.0001$) which began one day after the treatment and was maintained in the long term (D9: -24%, D11: -15%, D90: -15%).

Males	9 days	11 Days	90 Days
Control group			
Corticosterone (ng/ml)	5.26 ± 0.10	7.53 ± 0.10	22.52 ± 3.12
Triiodothyronine (pg/ml)	2.78 ± 0.11	4.13 ± 0.23	5.54 ± 0.13 #
Tyroxine (pg/ml)	8.10 ± 0.37	17.9 ± 0.70 #	16.44 ± 0.97
Oral breathing			
Corticosterone (ng/ml)	102.11 ± 11 *	142.33 ± 31 *#	27.77 ± 3.43 #
Triiodothyronine (pg/ml)	1.37 ± 0.29 *	3.16 ± 0.15 *#	4.02 ± 0.80*#
Tyroxine (pg/ml)	6.16 ± 0.26 *	15.15 ± 0.36 *#	14.45 ± 0.27 *

Table 5. Impact of early nasal obstruction on plasma corticosterone, triiodothyronine and thyroxine levels at 9, 11 and 90 days of age in male control and oral breathing animals. Values are means ± S.E.M (n = 7 rats/group/age). Analysis of t-test: * significant difference from control group at 9 and 11 days at $P < 0.05$. # significant difference from the previous day at $P < 0.05$.

In the female group (table 6) a significant specific increase of plasma corticosterone level was found for the three ages ($F = 16.34$, $P < 0.0001$).

Oral breathing was associated with a decreased level of plasma triiodothyronine ($F = 38.39$, $P < 0.001$).The reduction was -46% at D9 and 26% at D11.Table 6 shows that in females oral breathing was associated with a decreased level of plasma tyroxine ($F = 18.85$, $P = 0.007$) which began one day after the treatment (D9: -68%, D11: -11%). No differences were observed in the long term for triiodothyronine and tyroxine ($F = 12.80$, $P = 0.80$).

Females	9 days	11 Days	90 Days
Control group			
Corticosterone (ng/ml)	6.69 ± 0.11	5.12 ± 0.11	14.13 ± 3.71 #
Triiodothyronine (pg/ml)	2.75 ± 0.11	4.11 ± 0.23 #	5.54 ± 0.13
Tyroxine (pg/ml)	6.88 ± 0.59	16.14 ± 0.41 #	12.58 ± 0.48
Oral breathing			
Corticosterone (ng/ml)	40.86 ± 3.04 *	75.73 ± 4.95 *#	42.77 ± 0.01 *#
Triiodothyronine (pg/ml)	1.37 ± 0.29 *	3.16 ± 0.88 *#	4.02 ± 0.8 #
Tyroxine (pg/ml)	2.25 ± 0.46 *	14.39 ± 0.35 *#	12.43 ± 0.31

Table 5. Impact of early nasal obstruction on plasma corticosterone, triiodothyronine, thyroxine levels at 9, 11 and 90 days of age in female control and oral breathing animals. Values are means ± S.E.M (n = 7 rats/group/age). Analysis of t-test: * significant difference from control group at 9 and 11 days at $P < 0.05$. # significant difference from the previous day at $P < 0.05$.

4 – Discussion

Our results show that oral breathing caused early changes in craniofacial development, which began within one day after treatment and was maintained at least until adulthood. Oral breathing was associated with a decrease in the vertical development of the nasomaxillary complex (E-U line) and in the development of the longitudinal skullbase (A-I line) in both sexes. Our results show also that early bilateral nasal obstruction associated with oral breathing did not cause asymmetrical development of the skull. Indeed, during this period no change was observed in right (L1-O) and left (L2-O) maxilla. Oral breathing appears also to have no role in the growth of the posterior transverse diameter (X1- X2). These results showed that the nasal obstruction period was associated with delays in juvenile craniofacial developmental in males and females. Similar findings were reported by Yamada *et al.* (1997), who observed permanent craniofacial deformities after inducing nasal obstruction, in young Macaca monkeys (before and during puberal development). Yamada's model appears particularly interesting as the animals used are phylogenetically close to humans. They are limited, however, by the fact that genetic variability cannot be excluded.

The influence of oral breathing has also been claimed to explain the posterior rotation of the mandible, as reported in children affected by adenoid hypertrophy. Lessa *et al.* (2005) showed also that oral breathing was associated with an increased mandibular inclination, vertical growth pattern with changes in normal facial proportions, characterized by increased anterior lower facial height and decreased posterior facial height in oral breathing children.

In the long term, 90 days after the reopening of the nostrils, the results observed in males show that the delay of craniofacial growth observed during the period of nasal obstruction could not be corrected. Indeed, in males, oral breathing was associated with a long-term reduction in the nasomaxillary complex (E-U line) and in the skullbase longitudinal axis (A-I line). By contrast in female oral breathing rats a decrease in the

skullbase longitudinal axis (A-I line) was observed in the long term. These results show that in rats early oral breathing was associated with long-term sexual dimorphism. Several studies have shown a link between sex and craniofacial development. Kawashima (2002) found that pre-school boys with respiratory disorders during sleep presented a higher anterior lower facial height than girls. This was associated with a significantly higher percentage of nose breathing among girls than boys (Vig, 1998). It has been demonstrated that ovariectomy and orchiectomy induce bone loss, and that oestrogens and androgens are effective in the prevention of bone loss during adolescence (Fujita *et al.*, 2004). These results demonstrate that different sex hormones play an important role in craniofacial development.

To our knowledge no study has been made on craniofacial development during the period of nasal obstruction and 90 days after the reopening of the nostrils. This study provides information that early nasal obstruction associated with a switch to chronic oral breathing causes long term craniofacial growth retardation with a sexual dimorphism.

Nasal obstruction and the associated switch to temporary forced oral breathing were correlated with increased basal plasma levels corticosterone, (one day after treatment) that this result was maintained at long term (D90) only in females. This condition is known to cause an alteration in bone development.

Several studies have reported that glucocorticoids in growing animals reduce body weight and longitudinal bone growth (Arcus & Kagan, 1995 ; Fujita *et al.*, 2008). In our study, increased glucocorticoids resulted in a reduction in body weight, in skullbase longitudinal axis and in nasomaxillary complex. These findings are consistent with those of Fujita *et al.* (2004) who demonstrated that increased glucocorticoids resulted in a reduction in body weight and bone (mandibular) length. Davidovitch (1971) demonstrated also that excess glucocorticoids had suppressive effects on tibial growth. The cellular mechanisms by which glucocorticoids exert their effects on bone are complex with a range of direct and indirect effects on the cell types present in bone. All bone cells appear to express the classical glucocorticoid receptor. The most important effects appear to be direct action of glucocorticoids on osteoblasts to reduce their activity and cause their apoptosis and a stimulatory action on the activity of osteoclasts (Weinstein *et al.*, 1998; Weinstein, 2001).

The literature has shown that glucocorticoids, the end product of hypothalamic-pituitary-adrenal (HPA) axis activation, can inhibit the hypothalamic-pituitary-thyroid (HPT) axis (Kakucska *et al.*, 1995). Our current data would support this proposition, in that nasal obstruction induced increased corticosterone levels which were associated with lower plasma thyroid hormone (T3, T4) levels up to 90 days after the nostril reopening in males. Reduction of thyroid hormones can be explained also by other factors such as nutritional depletion (Hansen *et al.*, 2004).

The hypothalamic-pituitary-thyroid axis plays a key role in skeletal development, acquisition of peak bone mass and regulation of adult bone turnover. Childhood congenital and juvenile acquired hypothyroidism delayed severely skeletal development causing growth arrest and impaired bone maturation (Rivkees *et al.*, 1988).

These results are consistent with ours, which showed that the decreases in thyroid hormones were coupled with a delay of craniofacial growth. Our results showed that the delays observed in the young are maintained long-term in males. Oral breathing had a long term impact on craniofacial development in males which may be due to hypothyroidism which was maintained over the long term in these individuals.

Our results showed also that animals with oral breathing presented an impaired olfactory bulb development during the period of nasal obstruction (D9, D11) as well as in the long-term (D90). These results showed that in the rat, atrophy of the olfactory bulbs begins within one day after treatment (D9) and remains for the long term (D90).

Many studies have shown the impact of early nasal obstruction on development of the olfactory bulbs in rats. However, no study has shown that its impact is rapid and lasts through the long term. Gelhaye *et al.* (2006a) showned the impact of early nasal obstruction (D8) on the development of the olfactory bulbs at weaning (D21); Loranca & Salas (2001) have shown also that early nasal obstruction (D3) caused atrophy of the olfactory bulbs for up to 70 days. Our results showed that atrophy of the olfactory bulbs began within one day (D9) in males and two days (D11) in females after nasal obstruction. These results were maintained long term (D90) in both sexes.

Furthermore, spontaneous reopening of the nasal passage is not fully complete, therefore the olfactory bulb may not receive sufficient stimulation for normal responsiveness, as already mentioned above with respect to flaring. Two hypotheses may be advanced to explain these changes: a decrease in neoneurogenesis and / or increased apoptosis (Farbman *et al.*, 1988 ; Bauer *et al.*, 2003). Although all the nostrils

are open to D15, atrophy of the olfactory bulbs was maintained for up to 3 months. The accentuation of atrophy must involve other factors such as hormonal changes. Receptors for thyroid hormones and glucocorticoids are present on cells of the olfactory bulb from the early stages of development (Morimoto *et al.*, 1996 ; Galeeva *et al.*, 2002). Moreover, postnatal hypothyroidism can cause reduced proliferation, not only in the olfactory epithelium, but also in the olfactory bulbs (Paternostro & Meisami, 1991 ; Hoyk *et al.*, 1996).

Nasal obstruction is associated with the establishment of oral breathing, which causes short-term stress and disruption in the development of the rat. Indeed, our results have shown atrophy of the lungs. This atrophy may be explained partly by the fact that oral breathing is less effective than nasal breathing (Morton *et al.*, 1995) and partly by a lack of sensory stimulation, which resulted in a decrease in the number of cells in the pulmonary mucosa. To our knowledge, no studies have shown an impact of early oral breathing on lung development.

In conclusion, the present study has shown that the craniofacial development and the level of plasma corticosterone and thyroid hormones can be altered by temporary forced oral breathing. These hormonal changes may be also a primary factor explaining the change of craniofacial development. The observed changes began one day after nasal obstruction and were maintained over the long term. These results imply that the establishment of oral breathing in humans in pathological conditions (allergic rhinitis, rhinosinusitis, adenoid hypertrophy and nasal polyps) could have an affect over the long term. This shows the importance of treating nasal obstruction rapidly.

CHAPITRE III

Incidences d’une obstruction nasale précoce

sur le comportement sexuel

L’olfaction est avant tout le sens de l’individualisation chimique. Elle permet une analyse qualitative et quantitative à l’échelle de la molécule, bien que la discrimination olfactive ne coïncide pas toujours exactement avec la différenciation chimique. Un animal donné n’est pas toujours capable d’analyser un mélange de substances. On observe à ce sujet les plus grandes variations en fonction des espèces envisagées, chacune paraissant adaptée à la perception des odeurs qui lui sont nécessaires dans sa vie végétative et sexuelle. Par ailleurs l’apprentissage et l’expérience acquise paraissent jouer un grand rôle dans la gamme d’odeurs et dans le pouvoir de discrimination auxquelles un Mammifère est sensible. De plus la sensibilité olfactive n’est pas un phénomène physique simple, car elle se trouve également sous la dépendance du système nerveux central qui « interprète » les données objectives de l’appareil sensoriel périphérique.

Par ailleurs, les facultés olfactives sont très diversement partagées chez les Mammifères. Ces variations concernent aussi bien l’aspect quantitatif (seuils d’excitabilité) que l’aspect qualitatif de la perception (gamme d’odeurs perçues, pouvoir de discrimination et d’analyse d’odeurs complexes).

La constitution anatomique des fosses nasales et surtout de leur partie olfactive permet dans une certaine mesure de déterminer à priori leur acuité olfactive. Chez les vertébrés, les chimiorécepteurs sont situés dans l’épithélium qui tapisse la cavité des organes olfactifs. On peut diviser les Mammifères en 3 groupes : les anosmiques, les microsmiques et les macrosmiques. Dans le cadre de notre travail, seuls les Mammifères macrosmiques sont étudiés car l’olfaction dans ce groupe joue un rôle important. L’organe olfactif prend chez eux un développement considérable, de même que la portion correspondante du cortex cérébral. Chez certains animaux macrosmiques, la partie correspondant à l’olfaction atteint les 2/3 du cortex. L’organe olfactif logé dans le museau atteint une très grande complexité chez les marsupiaux, insectivores, carnivores, rongeurs où l’air inspiré parcourt un véritable labyrinthe.

1 – Le système olfactif des Mammifères

Le système olfactif se situe à l'interface entre l'environnement et le système nerveux central. Il est responsable du codage de l'information sensorielle provenant de milliers de stimuli odorants. Pour ce faire, l'information chimique doit être traitée tout au long de niveaux distincts. À chaque niveau, une représentation modifiée du stimulus olfactif est généré (Sobel *et al.,* 1998 ; Korsching, 2002 ; Leon & Johnson, 2003). Pour réaliser sa grande variété de fonctions, le système olfactif est composé de systèmes anatomiquement et fonctionnellement distincts : **l'épithélium olfactif, le bulbe olfactif et l'organe vomeronasal** (Halpern, 1987 ; Mori *et al.,* 1999 ; Keverne, 1999 ; Buck, 2000 **;** Firestein, 2001 ; Mombaerts, 2004).

Chez les Mammifères, **l'épithélium olfactif** recouvre la partie antérieure et dorsale des cavités nasales et est connecté au système nerveux central. Il est le siège de la transduction des signaux olfactifs en message nerveux. Chez les rongeurs, il tapisse des extensions de l'os éthmoïde qui forme des sinuosités. Celles-ci augmentent la capacité d'interaction et canalisent l'air. De ce fait, l'air passe d'abord dans la partie centrale et dorsale des sinuosités, ensuite dans les parties latérales et ventrales, sous la forme d'un flux laminaire, avant de sortir de la cavité nasale par la choane 3 (Kimbell *et al.,* 1997). Il est également recouvert d'un mucus, produit par des glandes particulières présentent dans la cavité nasale.

L'épithélium olfactif est constitué de trois types de cellule : des **cellules de soutien**, des **neurones sensoriels olfactifs** et des **cellules basales** (Moulton & Beidler, 1967).

Les neurones sensoriels olfactifs sont impliqués dans la réception des stimuli, la transduction et la transmission de l'information sensorielle (Meisami, 1989 ; Firestein, 2001 ; Mombaerts, 2004). Chez les rongeurs, on compte plusieurs millions de neurones sensoriels qui tapissent l'ensemble de la muqueuse olfactive (Ma *et al*., 1999). Les neurones olfactifs sont bipolaires et présentent une dendrite, qui se termine par une crinière de cils plongeant dans le mucus olfactif, et un axone, au niveau de leur pôle basal, qui projette jusqu'au bulbe olfactif dans des structures appelées glomérules. Le relais de l'information sensorielle est effectué par le **bulbe olfactif**.

Le bulbe olfactif est un organe pair situé dans la partie antérieure du cerveau. Il intervient dans le traitement des informations olfactives. Il reçoit des afférences de

l'épithélium olfactif et communique l'information sensorielle à des structures corticales et sous-corticales (Duchamp-Viret, 1999). Les axones des cellules du bulbe olfactif forment le tractus olfactif latéral (Figure 2C). Le bulbe olfactif est le siège d'une importante transformation des entrées sensorielles, pour l'essentiel mono-toniques (Friedrich & Laurent, 2001 ; Breipohl *et al.*, 2001) ajoutant des composantes spatiales et temporelles dans l'information sensorielle qu'il reçoit. Les neurones du bulbe olfactif empruntent le nerf crânien I et se projettent sur le cortex olfactif. De plus, des voies olfactives se rendent vers l'amygdale et l'hippocampe, des régions du système limbique impliquées dans l'émotion et la mémoire.

Chez les Mammifères macrosmiques, une structure olfactive accessoire se trouve dans la cavité nasale, **l'organe vomeronasal** ou **organe de Jacobson**. La structure de l'organe voméronasal et l'analogie de son revêtement avec la muqueuse olfactive donnent à penser qu'il s'agit d'un organe olfactif accessoire. Cet organe est constitué par 2 diverticules symétriques inclus dans le plancher des fosses nasales de chaque côté du septum nasal, en forme de cul-de-sac s'ouvrant chez la plupart des Mammifères dans le **canal de Stenson** ou **canal nasopalatin**. Chacun de ces diverticules est inclus dans une capsule protectrice de développement variable suivant les espèces (**cartilage de Jacobson**). Cette capsule est ossifiée chez quelques Mammifères, comme le Cobaye.
Le diverticule de l'organe de Jacobson s'ouvre par un canal à un niveau variable dans le **canal naso-palatin** ou **canal de Stenson**, vestige conservé de la disposition reptilienne après la constitution du palais mammalien, passant entre les maxillaires et les prémaxillaires. Il s'ouvre donc ainsi indirectement dans la cavité buccale. Ce n'est que chez les rongeurs que le canal s'ouvre dans la cavité nasale elle-même, près de la base du septum. Les cellules sensorielles de l'organe vomeronasal sont des cellules bipolaires, se terminant à la surface de l'épithélium par des cils (dendrites). Leurs axones se réunissent pour se joindre aux nerfs voméronasaux, traversant la plaque criblée et se terminent dans une partie distincte du lobe olfactif.

Chez le rat, **l'épithélium olfactif principal** et **l'organe voméronasal** apparaissent dès le 13ème jour du développement embryonnaire, tandis que **l'organe septal** commence son développement au 15ème jour du développement (Oikawa *et al.*, 2001). Celui - ci forme une portion d'épithélium sensoriel se détachant graduellement de l'épithélium olfactif principal. La muqueuse située entre ces deux territoires sensoriels

abandonne ses caractéristiques d'épithélium olfactif pour se transformer en une muqueuse de type respiratoire vers le 20ème jour postnatal. À la naissance, l'épithélium olfactif principal a terminé son développement alors que les organes accessoires continuent leur maturation pendant une partie de la période postnatale (Garrosa *et al.,* 1992 ; Giannetti *et al*., 1995).

Chez le rat, l'olfaction est fonctionnelle à partir de la 3ème semaine de gestation tandis que l'organe septal ne deviendrait efficient qu'après le sevrage et l'organe voméronasal resterait fonctionnellement immature pendant les premiers jours postnataux (Garrosa *et al.,* 1992 ; Giannetti *et al.,* 1995 ; Breipohl *et al.,* 2001).

2 – Olfaction et attraction sexuelle chez les mâles

Afin de maximiser le succès de la reproduction, les animaux ont développé des mécanismes neuronaux et endocriniens permettant de coordonner les efforts de sélection avec des conditions sociales et environnementales appropriées. Parmi les facteurs sociaux qui influent sur la fonction de reproduction chez les Mammifères, l'olfaction est probablement le plus répandu et le plus puissant. En effet, des signaux chimiques, appelés **phéromones sexuelles**, sont utilisés pour communiquer des informations spécifiques à l'espèce qui module le comportement de reproduction (Keller et *al.,* 2009) ou l'état physiologique de l'individu (Vandenbergh, 1969). Les phéromones jouent un rôle important dans la recherche, la rencontre, et la reconnaissance des sexes, notamment la détection de la femelle par le mâle (Kelliher & Baum, 2001). Elles jouent également de la même manière dans la reconnaissance de l'état sexuel de la femelle (**oestrus**).

Ainsi, par exemple, l'attraction exercée par une femelle sur un mâle adulte est liée à la présence, soit dans l'urine, soit dans les sécrétions vaginales (Aron, 1979) ou dans les sécrétions préputiales de la rate (Orsulak & Gawienowski, 1972) d'attractants sexuels (**phéromones sexuelles attractives**) qui peuvent agir à distance sur le mâle. Chez la femelle du hamster, c'est une protéine de 20 kDa issues des sécrétions vaginales, l'**aphrodisine**, qui joue le rôle de phéromone sexuelle attractive. Chez la souris *Mus musculus*, c'est un mélange de 2 composés volatils présents dans l'urine : la 2-(sec-butyl)-thiazolidine et la 2.3-déhydro-exo-brévicomine. C'est ainsi qu'un mâle, même

naïf, peut manifester sa préférence pour une femelle en oestrus, et donc réceptive (Carr & Caul, 1962 ; Hayashi & Kimura, 1974).

Powers *et al.*, (1979) ont établi que des hamsters mâles, tout en conservant leur aptitude copulatoire après anesthésie de la muqueuse olfactive au sulfate de zinc, n'étaient plus attirés par des sécrétions vaginales répandues dans leur cage. Or ces mâles apparaissaient capables de copuler lorsqu'ils étaient mis en présence d'une femelle, sans doute parce qu'ils étaient encore sensibles, par la voie du système olfactif accessoire aux attractants sexuels de la femelle. Ce comportement a été aboli après section du nerf voméronasal. D'après Vandenbergh (1988), lors de l'activité sexuelle, les phéromones stimulent les terminaisons neuronales de l'organe voméronasal. L'information parvient aux bulbes olfactifs accessoires qui la transmettent directement à l'hypothalamus *via* l'amygdale. Ainsi, en transmettant des informations à travers le **bulbe olfactif accessoire**, l'organe vomeronasal fournit des informations sur la situation sociale et sexuelle d'autres individus au sein de l'espèce (Doving & Trotier, 1998). Toute fois, des données récentes suggèrent que l'organe voméronasal n'a pas une fonction exclusive à l'égard de la reconnaissance des phéromones sexuelles, mais il répond également à d'autres molécules, au moins chez les rongeurs (Luo *et al.*, 2003, Brennan & Keverne, 2004).

Par conséquent, dans le cadre du comportement sexuel, la perception olfactive s'effectue à distance grâce à des substances volatiles (phéromones sexuelles attractives) agissant sur la muqueuse olfactive, mettant alors en jeu le système olfactif principal, ou par voie de contact, sous l'effet de substances non volatiles dissoutes dans la salive (**phéromones sexuelles de contact**), et venant agir sur le système olfactif accessoire en impressionnant les récepteurs de l'organe voméronasal. L'attraction du mâle, préalable à l'accouplement, résulte donc bien de la perception, à distance et par contact, de signaux olfactifs émis par la femelle.

Mais pour q'une odeur soit perçue, les particules odorantes doivent nécessairement atteindre la muqueuse olfactive. L'analyse électrophysiologique de la sensation olfactive a montré que le contact direct des molécules odorantes avec les microvillosités de la cellule olfactive est indispensable. Le stimulus provient donc d'éléments matériels en phase gazeuse transportés au niveau de l'organe. Cela implique avant tout, la volatilité de la substance elle-même, en fonction de la température. Il faut de plus que la substance soit soluble dans l'eau et les lipides, afin de pouvoir entrer en

contact avec la muqueuse et les poils olfactifs de ses cellules sensorielles. Il existe plusieurs voies d'entrée des particules volatiles dans les **fosses nasales**.

On distingue :

* La **voie orthonasale** (voie principale) lorsque les molécules chimiques pénètrent dans la cavité nasale par les narines. Cet air est immédiatement réchauffé et humidifié dans la partie antérieure des fosses nasales, au niveau des maxillo-turbinaux. Les filets d'air sont alors dirigés vers les ethmo-turbinaux par des dispositifs anatomiques très précis chez les Mammifères macrosmatiques où tout le volume d'air inspiré entre en contact soit directement, soit par diffusion, avec les parois olfactives. Les particules vont s'y déposer avec d'autant plus de facilité que la muqueuse olfactive est humidifiée par des sécrétions séreuses.

* La **voie rétronasale** lorsque les molécules exhalées au cours de la mastication atteignent l'épithélium olfactif par le nasopharynx. Dans ce cas, les stimuli chimiques sont transportés par le flux expiratoire suivant la déglutition. Ainsi les odeurs alimentaires diffusent de la bouche vers le pharynx, d'où l'air expiré les transporte vers les fosses nasales ; cette stimulation constitue la composante olfactive du goût, les deux sens étant ainsi étroitement associés sur le plan physiologique.

* La **voie internasale** lorsqu'une molécule odorante passe d'une narine à l'autre par l'intermédiaire du nasopharynx.

* La **voie bucco-nasale** lorsque les molécules pénètrent dans la cavité nasale par le conduit naso-palatin. Chez le rat, cette voie reste effective tant que l'extrémité buccale du conduit naso-palatin est ouverte et interviendrait surtout au moment des évènements alimentaires.

Certains Mammifères peuvent utiliser les variations de l'intensité d'une odeur pour tirer des informations quant à la direction et la distance d'une source odorante (Rajan *et al.,* 2006). Les inspirations successives (flairage), permettent d'augmenter le débit aérien et donc la probabilité d'impact des molécules sur le système olfactif, ce qui accroît la perception de l'environnement olfactif (Laing, 1982).

3 – Obstruction nasale et attraction sexuelle chez les mâles

Nous venons de voir dans les chapitres précédents que l'obstruction entraîne à court et à long terme des modifications morphologiques.

Les animaux expérimentaux présentent à l'âge adulte des bulbes olfactifs significativement plus petits que ceux des animaux contrôles (cf. articles 3 et 4). Or les bulbes olfactifs jouent un rôle important dans le cadre de l'olfaction chez les Mammifères. Par ailleurs, ***nos résultats*** *(table 1) montrent que l'ouverture des narines à l'âge adulte chez les mâles est significativement plus petite chez les animaux expérimentaux (F = 7.22, P = 0.004).*

Rats	Mâles	Femelles
Contrôles	**238.25 ± 0.54**	**218.50 ± 0.77**
Expérimentaux	**214.50 ± 0.95***	**207.13 ± 0.53**

Table 2 : Ouverture des narines (en µm) chez des rats adultes de 90 jours. * significativement différent du lot contrôle à P = 0.004.

Suite à ces observations**,** notre étude, présentée dans **l'article 5**, a été donc conçue pour tester les effets à long terme de l'obstruction nasale sur la capacité olfactive des mâles à l'âge adulte à différentier des odeurs de femelles adultes. Des tests comportementaux et des analyses physiologiques ont été réalisés 90 jours après l'ouverture des narines et le rétablissement de la respiration nasale. Une série de 3 expériences, effectuées dans des labyrinthes, nous a permis d'examiner les réponses de rats mâles aux odeurs de femelles sexuellement réceptives.

Les mâles témoins et contrôles ne montrent aucun problème de préférences pour des odeurs des femelles en oestrus. Pendant les tests de choix olfactifs, les stimulations olfactives entraînent une élévation rapide du taux plasmatique de testostérone mais également du taux plasmatique de corticostérone en relation avec le comportement exploratoire dans un milieu non familier. Les mâles expérimentaux à l'âge adulte passent plus de temps à explorer les enceintes expérimentales. Ils présentent des taux plasmatiques de corticostérone significativement supérieur aux taux observés dans les

mêmes conditions chez les rats témoins et contrôles. De plus les rats expérimentaux ne présentent aucune préférence olfactive entre des odeurs de femelles adultes non réceptives ou réceptives sexuellement. Le taux plasmatique de la testostérone chez ces mâles, contrairement aux autres mâles, ne varie pas sous l'influence des odeurs des femelles, et reste significativement plus faible par rapport aux autres congénères. Par contre les niveaux plasmatiques de cholestérol et des triglycérides sont significativement plus élevés chez ces mâles expérimentaux. Par ailleurs, nos expériences montrent que ces mâles expérimentaux ont une préférence pour l'odeur des femmes adultes qui ont reçu au stade néonatal, comme eux, une obstruction nasale. Ces résultats montrent bien que les mâles sont capables de différencier l'état physiologique des congénères. On peut penser d'après nos résultats, que nos mâles n'ont pas encore eu un éveil de la libido (maturation sexuelle retardée) ou que le système olfactif (bulbe olfactif, organe voméronasal) n'est pas réceptif aux phéromones attractives sexuelles des femelles.

Article 5

Prosexual odour differentiation and physiological profile in adult male rats after a neonatal, short term, reversible olfactory deprivation

S. Padzys, J.M. Martrette, S. N. Thornton, Marie Trabalon

(soumis)

1. Introduction

Under normal conditions lack of olfaction is generally associated with the obstruction of the nasal cavities (Seiden & Duncan, 2001). Our previous study revealed indeed that nasal obstruction induced in eight-day old rats led to activation of the stress response with increased corticosterone as the final effecter (Gelhaye et al., 2006 a, b). The literature shows that increased plasma levels of corticosterone are associated with the expression of anxiety-related behaviours such as hyperactivity (Cao et al., 2007), with modifications of novelty-seeking behavior (Alemany, 2008; Gelhaye et al., 2006b, 2011), and social play in juvenile rats (Beatty & Costello, 1983; Risser & Slotnick, 1987).

Through olfactory deprivation and activation of the HPA axis, early nasal obstruction could disrupt on the one hand establishment of exploratory behaviour, a preliminary necessary for the emancipation and the dispersion of young mammals, and on the other hand the hormonal homeostasis of young individuals. Nasal obstruction generates numerous effects on the olfactory bulb, including reduction of its volume (Gelhaye et al., 2006a) and a variety of neurochemical and functional changes (Brunjes, 1994). However, in spite of the blockage of the nasal cavities, odorants could, theoretically, reach the olfactory epithelium by the nasopharynx and this retronasal perception could mediate odour-guided behaviours in rodents (Coppola et al., 1994; Chapuis et al., 2007).

In many species, including humans, chemosensory stimuli function as social cues that impact reproductive hormones and behaviour (Doty, 2001; Jacob et al., 2001; Wirsig-Wiechmann, 2001). In rodents, female odours (or pheromones) activate neurons in limbic circuits mediating male reproductive behaviour and elicit gonadal steroid release in sexually naïve males (Meredith, 1998). The expression of reproductive behaviour in sexually naïve male Syrian hamsters is absolutely dependent on female pheromones present in vaginal secretions and their transduction by the vomeronasal system (Meredith, 1986; Meredith & Howard, 1992). In male hamsters and rats, exposure to female pheromones elicits a rise in testosterone within 60 min (Richardson et al., 2004; Wood et al., 2004). Furthermore, female chemosensory stimuli can be used to establish a classically conditioned endocrine response to a neutral stimulus, confirming their roles as unconditioned stimuli for evoking reproductive responses in males (Graham & Desjardins, 1980).

Olfactory recognition appears often to be important in establishing the bond between mammalian individuals. Young distinguish their own mother from other females by recognizing her distinctive odor (Leon & Moltz, 1971; Brunjes & Alberts, 1979; Brown, 1982; Polan & Hofer, 1998). Early imprinting with the correct odour may influence not only the young animal's future recognition of and relations with its mother, but also its selection of a mate having a similar odour when it becomes adult (Loranca & Salas, 2001 ; Bakker et al., 1996; Gasperin-Estrada et al., 2008). A mammalian pheromone used for individual recognition may volatilize directly from the body of the animal, or it may be deposited onto a substratum as a scent mark. A scent mark has the advantage of allowing an animal to identify the previous presence of either a known or an unknown individual of the same or a different species in a particular area. These scent marks may act as loci for the general exchange of information such as individual identity, as well as age, sex, breeding condition, and social status of the marking animals (Kalkowski, 1967; Müller-Schwarze, 1971, 1972; Johnson, 1973).

Precocious olfactory experience has a very important role to play in rat neurobehavioral development (Sczerzenie & Hsiao, 1977; Coopersmith & Leon, 1984; Hongo *et al.*, 2000). Learning of the maternal odour is critical for the young's survival as she is, at least initially, the unique source of nourishment, heat and protection (Sullivan, 2003). Newborns must learn rapidly not only their mother's odour but also that of the nest and then their littermates in order to better orient their movements both

within and outside the nest. In preventing these olfactory signals bilateral nasal obstruction could lead to partial social isolation and thus be also a sensorial stress. In fact during postnatal cerebral development, rat pups have only olfactory input to orientate themselves as hearing and vision are acquired from the 10^{th} to the 14^{th} day after birth. Those pups with nasal obstruction during this initial period have only the senses of touch and temperature to inform them of their environment.

The aim, therefore, of the present study was to evaluate the effect of early, reversible, nasal obstruction, giving a short term olfactory deprivation period on sexual differentiation of odours and potential partner selection in adult male rats. We have used uniquely odour cues with no physical interaction tests as it is well known that rats make ultrasonic vocalization during mating (Barfield & Thomas, 1986; Matochik & Barfield, 1991). The effects of early olfactory deprivation on the stress response (corticosterone), on plasma levels of sexual hormones (progesterone, oestradiol and testosterone), and on biochemical markers (glucose, proteins, lipids) were studied also in adult rats.

Our hypothesis was that early short-term olfactory deprivation would have a significant effect of some parameters of exploratory and prosexual behaviour. We also investigated the functional impact of early olfactory deprivation on olfactory abilities. Nest recognition and choice of sexual odour partner (oestrus and anoestrus females) were therefore investigated in a two-choice situation. Vertebrates are frequently characterized as being able to recognize the physiological status other individuals, so the choice of odours from untreated, control, or early nasally obstructed females were therefore investigated in a three choice situation.

2. Methods

2.1. Animal care

Male and female Wistar rats (origin IFFA- CREDO) were used in these experiments. These pups were born in the laboratory from 15 litters, culled to 10 pups per litter (5 males and 5 females) to ensure normal body growth. We used three male from each litter for each of the biological and behavioural tests. We used all females from each litter for the behavioural tests. The animals were housed in standard cages under controlled temperature conditions (22 ± 1°C). Food (pellets of 12 mm, Harlan

Interfauna Iberica SA) and water were available *ad libitum* throughout the experiment. From birth, the rats were kept on a reversed 12:12 light-dark cycle (dark period 08:00-20:00h).

2.2. Nasal obstruction procedure

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (no. 85-23, revised 1996), the recommendations of the European Community Council for the Ethical Treatment of Animals (no. 86/609/EEC) and the regulations of the University of Nancy 1. All efforts were made to minimize animal suffering.

At 8 days of age, the litters were first anesthetized by hypothermia (10 min at -18°C), then weighed and then semi randomly divided into one untreated group, one control group (sham) and one experimental group (nasal obstruction or NO). Bilateral nasal obstruction resulting in forced oral breathing was performed in experimental animals (15 per group and sex from five litters) as described previously by Gelhaye et al. (2006a, b; 2011) and Padzys et al. (2011). The selected method consisted in cauterizing the external nostrils, which is the most common and simple procedure allowing reversible nasal obstruction in neonates. The tissue surrounding the external nostrils was burned by placing a surgical cauterizing instrument (1 mm in diameter) on the nostrils, consequently occluding the orifice of the nostrils without mechanical or chemical damage to the olfactory mucosa. This procedure induced complete nasal obstruction between D8 and D11 with 100 % of the nostrils reopened at D15. This was tested regularly with a weak soap solution applied to the nostrils. Small bubbles were observed once the nostrils started to open at the end of D11.

In the sham group (SH), the nostrils were not sealed but the cauterizing instrument was placed about 1-2 mm above each nostril (15 per group and sex from five litters). After cauterization, the nostrils were washed with chlortetracycline (Aureomycine Evans 3%) to prevent infection. In the untreated group (UT) the rats were anesthetized only (15 per group and sex from five litters).

The pups were warmed (37°C) for 30 min and returned to their mothers. The pups were weaned at 25 days of age and then housed in a cage with 2 conspecifics of the same sex and treatment. Animals were left undisturbed until the onset of behavioural testing and sample collection at 90 ± 4 days after post-reopening of the nostrils (D110). As shown schematically in Figure 1.

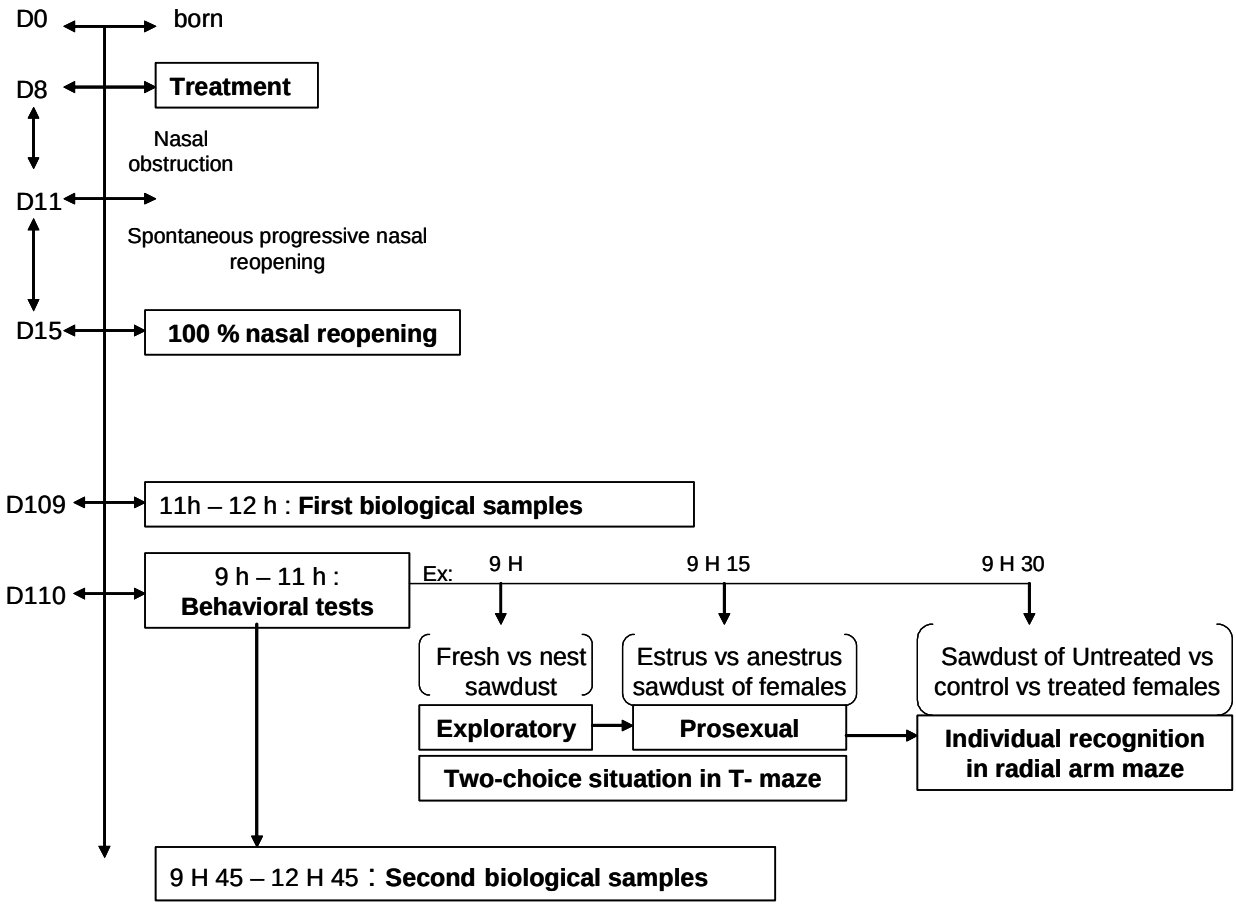

Figure 1: Time line of experimental protocol.

2.3. Behavioural analysis: *exploratory and odour partner preference with no physical interaction tests*

For behavioural observations we used a T-maze and a radial arm maze. The T-maze presented a start arm connected to two goal arms of equal dimensions (30 cm x 10 cm x 10 cm). The maze was constructed of Plexiglas, with a guillotine door separating the start box from the main stem of the maze. An experimental box (40 cm x 20 cm x 15 cm) was situated at the end of each goal arm and closed by a door with holes in it which allowed stimulation by smell. The radial arm maze was an array of three arms (30 cm x 10 cm x 10 cm) radiating from a central starting area. At the end of each arm we placed an experimental glass box (40 cm x 20 cm x 15 cm). In all cases, 45 ± 5 g of sawdust were placed in the experimental boxes and each peripheral box, placed at random, were presented only once. Each experimental box was tested only once. The experimental set-up was washed with an acetone-water solution (5%) between each test to obviate

possible biasing effects of odour by the previous rats nest sawdust. A total of three tests were conducted for each male.

- Eploratory behaviour during a two-choice situation in the T-maze. In order to evaluate exploratory behaviour, adult male rats were observed in the two-choice situation “fresh sawdust *versus* nest sawdust”.

- Prosexual olfactory choice during a two-Choice situation in the T-maze. Immediately after the exploratory test, the male was placed in another T-maze with “nest sawdust of an UT oestrus female *versus* nest sawdust of an UT anoestrus female”. In females, oestrus cycles were tracked by examination of morning vaginal smears, and only those rats showing three consecutive 4-day cycles of oestrus were used. Nest sawdust of females only at the oestrus and anoestrus stages were collected at the beginning of behavioural test.

At the beginning of the T-maze test, sawdust was placed in each experimental box and a male was placed in the start box. Five minutes later, the guillotine door was opened allowing the male to move freely in any arm. The observation period began when the rat entered the start arm of the T-maze and the guillotine door was closed behind it during the 10 min test.

The latency of the first choice (defined by the first contact between an animal and a lateral box), and the time spent with sniffing in each arm of the T-maze were recorded.

– Individual recognition during three-Choice situation in radial arm maze. Immediately after the behavioural observations in the T-maze, male was placed in the central box of the radial arm maze and allowed to choose between a box with “nest sawdust of UT female”, a box with “nest sawdust of a SH female”, a box with “nest sawdust of an NO female”. Females are the same age as the male, 110 days.

The behavioural observation began the second the experimental rat touched the substratum of the central box and the tested rat was observed for 10 min after the first contact with one test box. The latency of the first choice (defined by the first contact between an animal and an experimental box), and the time spent with sniffing in each arm of the radial maze were recorded.

The behaviour of all males during the three behavioural tests was videotaped with video tracking equipment and analyzed with the Smart-MA programme (Smart Panlab,

Bioseb, France). The events were later quantified by a blind tester. To minimize the influences of possible circadian changes on behaviours, untreated, sham and OB animals were alternated for observation. They were observed at the same time of day (between 9:00 AM and 11:00 AM). The apparatus was maintained in the same position in the room throughout the duration of the study.

2.4. Sample collection

First sample: For hormone assays, rats were anaesthesia 24 hr before behaviour tests and intracardiac blood samplings (500 – 1000 µl) were taken between 11h and 12h for hormonal measurements. Blood was collected within 1-2 min into sterile heparinised syringes fitted with a 26-G needle. Plasma was immediately separated by centrifugation at 4°C (15 min at 3000 rpm) and the extracts aliquoted and stored at -36°C until the time of assay (corticosterone and testosterone).

Second sample: Immediately after the end of the behavioural observations, rats were anaesthesia, weighed and intracardiac blood samplings with same method. Plasma was aliquoted and stored at -36°C until the time of the assay (biochemical and hormones).

2.5. Hormone assays

Corticosterone, progesterone, 17β-oestradiol and testosterone concentrations were measured without an extraction procedure, using a commercially available EIA kit and performed according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentration of hormones in plasma samples was calculated from a standard curve and expressed as ng/ml for corticosterone and ng / ml for sexual hormones. The intra- and inter-assay coefficients of variation were under 8.4 % and 13.1 % respectively for corticosterone, 9.2 % and 7.4 % respectively for progesterone, 7.6 % and 8.3 % respectively for 17β-estradiol, 10.8% and 14.6% respectively for testosterone.

2.6. Biochemical assays: determination of glucose, protein and lipids levels

Concentration of blood glucose was determined using a colorimetric method after enzymatic oxidation in the presence of glucose oxidase (Glucose-test, Randox, UK). The hydrogen peroxide that forms then reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as indicator.

Protein content in 10 µl of plasma samples was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Triglycerides were determined using a colorimetric method after enzymatic hydrolysis with lipases (Triglycerides-test, Randox, UK). The indicator was quinoneimine formed from hydrogen-peroxide, 4-aminophenazone and 4-clorophenol under the catalytic influence of peroxidase.

Cholesterol was determined using colorimetric method after enzymatic hydrolysis and oxidation (Cholesterol-test, Randox, UK). The indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

2.7. Statistical analysis

The results were expressed as group means ± SE. Statistical analysis was performed by means of statistical software (Statview V5.0, Abacus concepts Inc., Berkeley, CA). Statistical analysis was carried out using the experimental condition (UT, Sham or NO) and the two or three choice situation as factors. Therefore, a two way ANOVA was used. The same procedure was used for: the time spent in each arm of the T-maze, the time spent in each arm of the radial arm maze; corticosterone and testosterone levels before and after the behavioural tests. In this last case, the PLSD Fischer test was used to establish the inter-group comparison. Concerning the other physiological data, group differences were determined using analysis of variance (one way ANOVA). Analysis of specific mean comparisons were then made using PLSD Fischer test. In all cases, the differences were considered significant at $P < 0.05$.

3. Results

3.1. Behavior tests

3.1.1. Exploratory behaviour during two-choice situation in T-maze

During the first test, “fresh sawdust *vs* nest sawdust”, no differences ($F = 1.12$, $P = 0.22$) were observed between the three groups of males for latency of the first choice. Rats choose more quickly the fresh sawdust box (7.3 ± 1.5 sec) versus 14.5 ± 0.9 sec for “nest sawdust” ($F = 2.74$, $P = 0.03$).

The time spent in each arm (Fig. 2) was comparable with the three groups: 100 sec in “fresh sawdust” and 70 sec in “nest sawdust” (F = 1.85, P = 0.18). The males spend approximately 80 % of time (428 sec) to explore the T-maze.

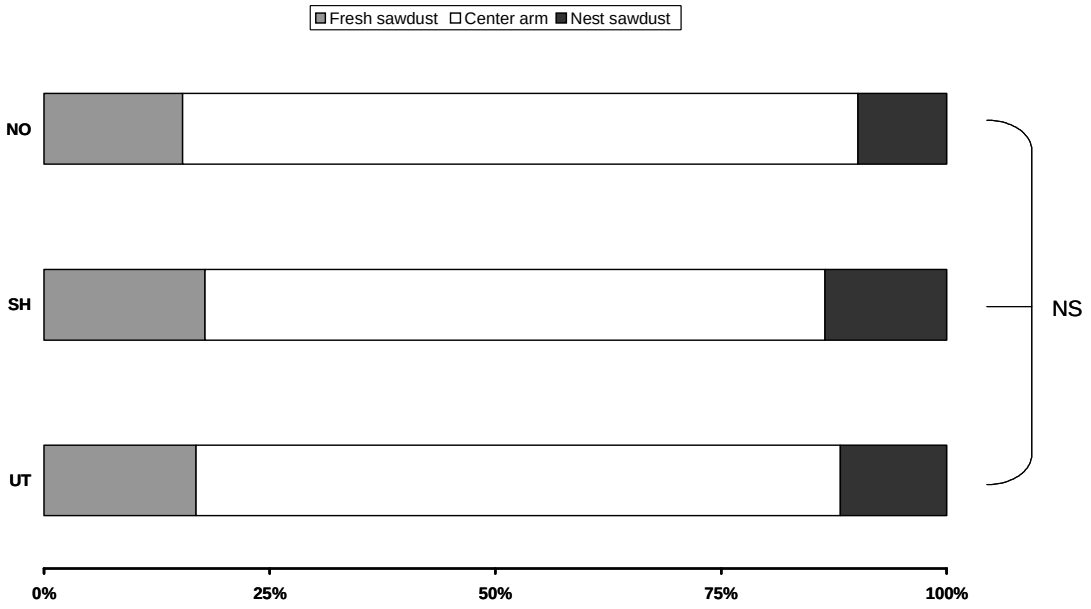

Figure 2: Impact of early nasal obstruction on male rats’ behaviour in a two-choice situation: “fresh sawdust vs nest sawdust”. Distribution of the time spent in each arm of the T-maze with untreated group (UT), sham group (SH), and animals with nasal obstruction at 8 days (NO). Values are means ± SE (n = 15 rats / group). Analysis of two-way ANOVA summary: NS not significantly different

As far as sniffing behaviour was concerned there were no differences between the groups (UT: 9.4 ± 1.6; SH: 9.4 ± 1.9; OB: 9.4 ± 2.8).

3.1.2. Prosexual behaviour during two-choice situation in T-maze

The latency of the 1st choice was comparable when SH and UT males had to choose between “nest sawdust of oestrus females *vs* nest sawdust of anoestrus females”: 10.2 ± 0.7 sec for “oestrus sawdust” and 9.84 ± 1.04 sec for “anoestrus sawdust” (F = 1.59, P = 0.82). NO males put significantly more time before going into the box of the “sawdust oestrus females”: 16.3 ± 1.4 sec *vs* 10.2 ± 0.7 sec for SH and UT males (F = 4.01, P = 0.02).

Fig. 3 shows that the induction of nasal obstruction affected the time repartition inside the maze with “nest sawdust oestrus female *vs* nest sawdust anoestrus female” (F = 51.83, P = 0.0001). NO animals spent less time in the nest sides compared to other animals: 139 ± 24 sec in “oestrus sawdust” and 105 ± 11 sec in “anoestrus sawdust” (P = 0.01). This significant difference was associated with a greater time spent in the centre arm (356 ± 29 sec). Sham and untreated rats spent significantly more time in the box of “oestrus females”: 294 ± 14 sec for SH males and 324 ± 32 sec for UT males (P < .001).

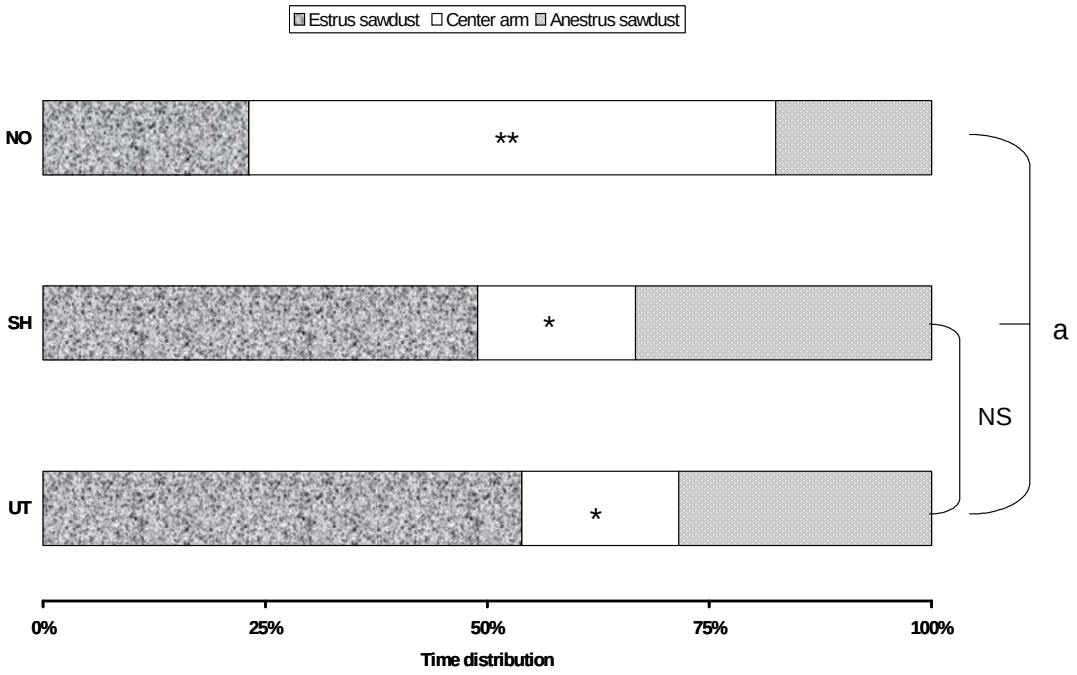

Figure 3: Impact of early nasal obstruction on male rats’ behaviour in a two-choice situation: « nest sawdust oestrus untreated female vs nest sawdust anoestrus untreated female ». Distribution of the time spent in each arm of the T-maze with untreated group (UT), sham group (SH), and animals with nasal obstruction at 8 days (NO). Values are means ± SE (n = 15 rats / group).
Analysis of two-way ANOVA summary (a): treatment effect: F = 173.66 at two degrees of freedom P < 0.0001; time spent in each arm: F = 51.83, P < 0.0001 at two degrees of freedom; treatment*time: F = 3.52, P = 0.05 at two degrees of freedom. NS not significantly different; * significantly different at P = 0.01; ** significantly different at p < 0.001.

There were no differences in sniffing behaviour between the groups (UT: 17.8 ± 2.1; SH: 18.6 ± 1.9; OB: 17.0 ± 3.9).

3.1.3. Social recognition during three-Choice situation in radial arm maze

During the three-choice test, no differences were observed between the three groups of males for latency of the first choice: 18.1 ± 1.3 sec for untreated rats, 17.9 ± 1.2 sec for sham rats, and 20.5 ± 2.1 sec for NO rats (F = 1.77, P = 0.14).

Fig. 4 shows that differences were observed between the three groups of males for time spent in each arm (F = 99.78, P < 0.0001). NO males spent comparable time in the "sawdust untreated" and "sawdust sham" (80 ± 20 sec) and significantly (P = 0.001) more time in the box of "sawdust NO females" (174 ± 25 sec) and the centre of the radial arm (266 ± 32 sec). Sham and untreated rats spent significantly less time in the box of "NO sawdust females": 130 ± 20 sec versus 270 ± 19 sec for UT females (P = 0.01).

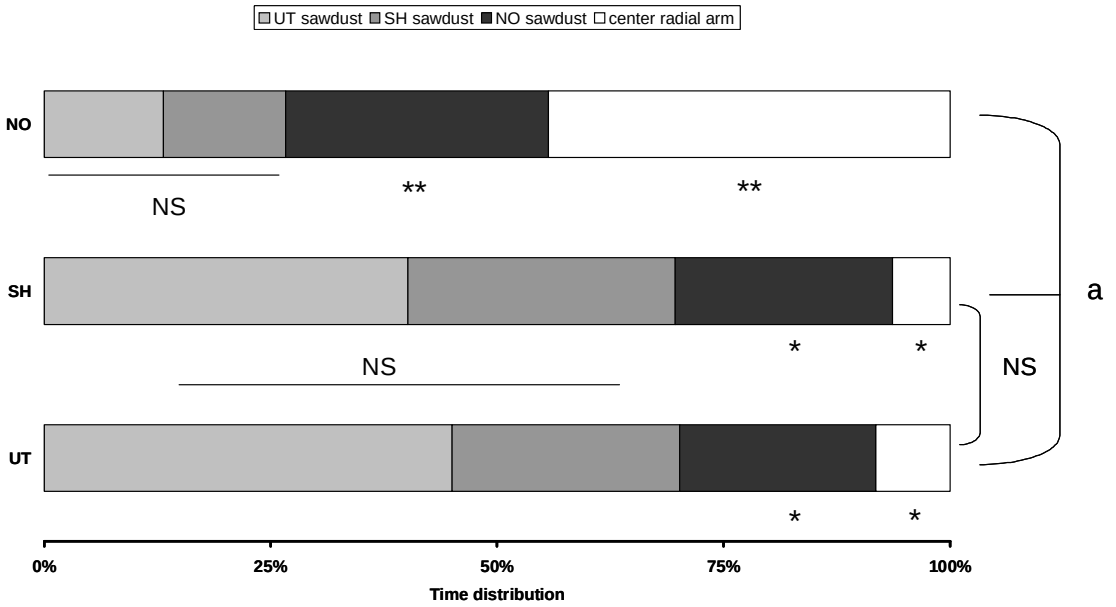

Figure 4: Distribution of the time spent in each arm of the radial arm maze with untreated group (UT), sham group (SH), and animals with nasal obstruction at 8 days (NO) in a three-choice situation: « nest sawdust untreated female (UT) versus nest sawdust sham female (SH) versus nest sawdust NO female » (n = 15 rats / group).
Analysis of two-way ANOVA summary: treatment effect (a): F = 73.95 at two degrees of freedom P < 0.0001; time spent in each arm: F = 99.78, P < 0.0001 at three degrees of freedom; treatment*time: F = 13.54, P = 0.001 at three degrees of freedom. NS not significantly different; * significantly different between different arms at P = 0.01 in SH; ** significantly different from SH and UT males at P= 0.001.

No differences in sniffing behaviour between the groups were observed (UT: 20.9 ± 1.7; SH: 19.7 ± 1.7; NO: 18.3 ± 2.7).

3.2. Body weight and Biochemical assay

Before the treatment, at 8 days of age, the weights of the three pups were not significantly different: 17.8 ± 0.4 g (F = 3.80, P = 0.18). Table 1 shows that the weights were similar at adulthood: 405 ± 13 g (F = 1.86, P = 0.17).

No differences were observed in male plasma glucose (F = 1.07, P = 0.35) and protein levels (F = 1.17, P = 0.32) between nasal obstruction, sham and untreated rats (Table 1).

Levels of triglycerides and cholesterol (Table 1) in NO male rats were significantly higher than sham and untreated males (F = 4.51, P = 0.02 and F = 3.26, P = 0.05, respectively).

Groups	Body weight (g)	Glucose (mg/dl)	Proteins (mg/ml)	Triglycerides (mg/dl)	Cholesterol (mg/dl)
Untreated	401 ± 11	235 ± 7	151 ± 6	93 ± 3	115 ± 10
Sham	421 ± 19	236 ± 12	155 ± 7	99 ± 5	139 ± 20
Oral breathing	394 ± 10	232 ± 5	132 ± 10	146 ± 10[a]	195 ± 15[a]
Mean	405[NS]	234[NS]	146[NS]	113*	150*

Table 1: Body weight, plasma glucose, proteins and lipid levels at 110 days of age in male rats with and without neonatal nasal obstruction.
Values are means ± SE. n = 15 rats per group. Analysis by ANOVA: *triglycerides: F = 3.26 at two degrees of freedom = $p<0.05$; * cholesterol: F = 4.51 at two degrees of freedom = $p<0.05$; [a]$p<0.05$ *versus* untreated and sham. NS = not significant.

3.3. Hormonal assay

3.3.1. Corticosterone

The concentration of corticosterone (Fig. 5) was significantly higher in NO rats before and after behaviour tests (F = 6.80, P < 0.0001). Short term nasal obstruction produced a significantly increase in plasma corticosterone levels compared with sham and untreated males. As shown in fig. 5, behavioural tests produced a significant increase in plasma corticosterone levels in the three groups of males (F = 25.29, P < 0.0001).

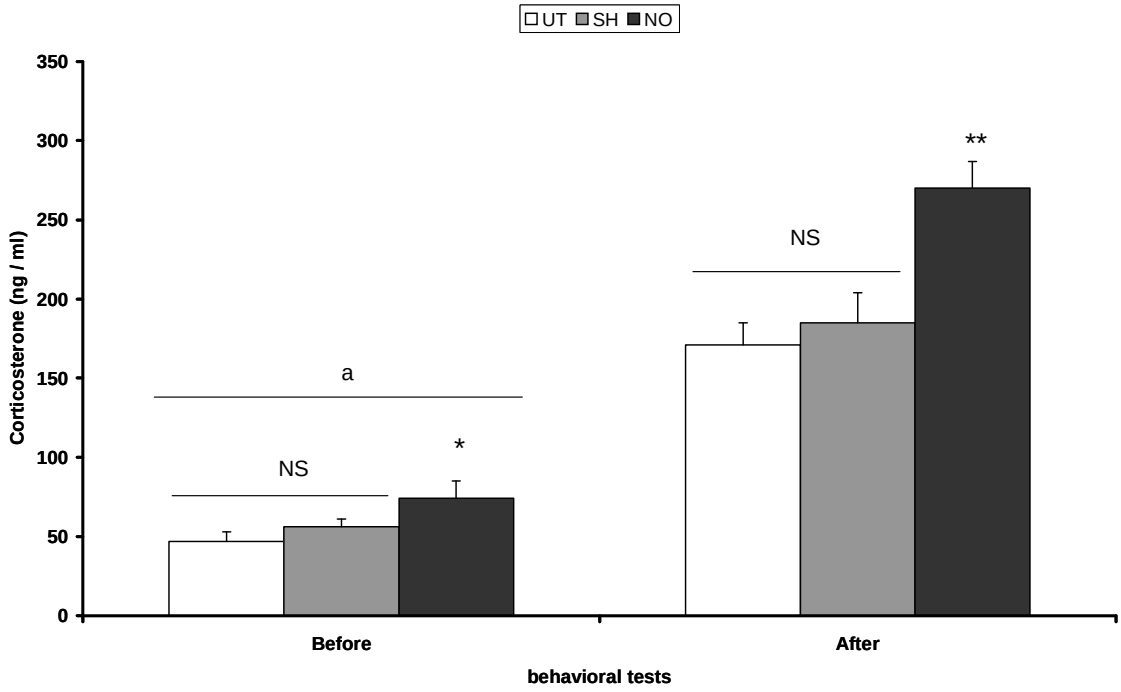

Figure 5: Impact of early nasal obstruction on plasma corticosterone level before and after behaviour tests in untreated (UT) group, sham group (SH) and animals with nasal obstruction at 8 days (NO). Values are means ± SE (n = 15 rats per group). Analysis of two-way ANOVA summary (a): treatment effect: $F = 4.75$ at two degrees of freedom $P < 0.05$; time: $F = 25.29$, $P < 0.0001$ at two degrees of freedom; treatment*time: $F = 6.80$, $P = 0.01$ at two degrees of freedom. NS not significantly different; * significantly different from UT and SH rats at $P < 0.05$; ** significantly different from UT and SH rats at $P < 0.01$.

3.3.2. Sexual hormones

Fig. 6 shows that plasma testosterone levels were significantly different between the experimental groups before and after the behavioural tests ($F = 23.99$, $P < 0.0001$). The level of testosterone was significantly lower in NO rats before tests: 2.1 ± 0.5 ng / ml versus 3.5 ± 0.5 ng / ml for SH and 3.8 ng / ml for UT rats ($P = 0.01$).

The level of plasma testosterone increased significantly ($P = 0.003$) after the behavioural tests in SH (7.5 ± 1 ng / ml) and in UT rats (7 ± 1 ng / ml).

The level of plasma testosterone did not vary in NO rats between before and after behavioural tests: 2.5 ± 0.7 ng / ml after tests.

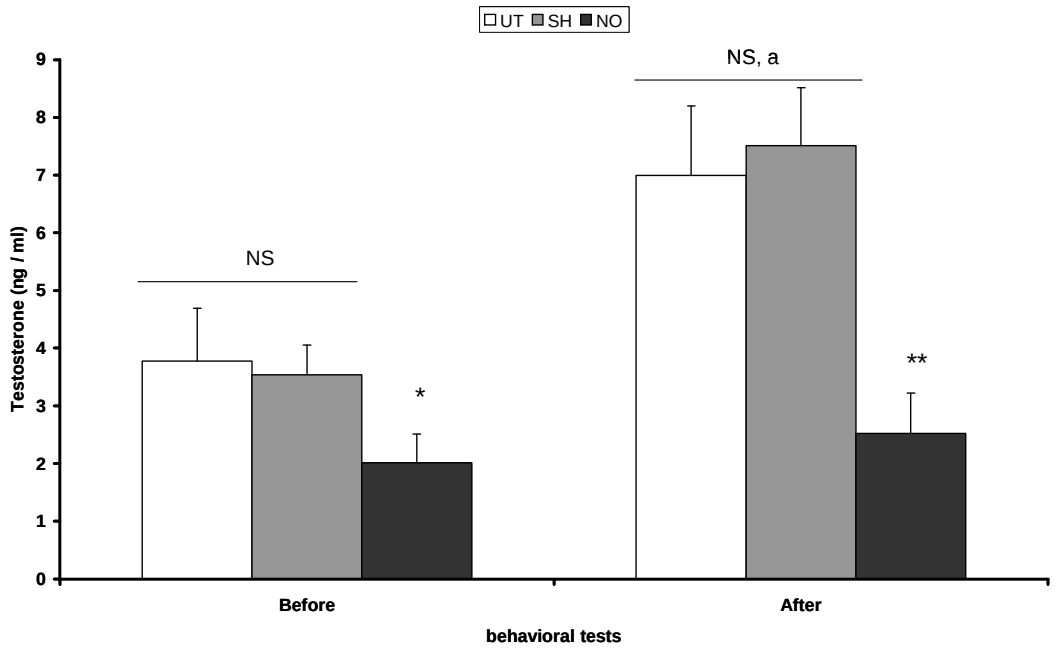

Figure 6: Plasma testosterone levels in untreated (UT) group, sham group (SH) and animals with nasal obstruction at 8 days (NO) before and after behavioural tests. Values are means ± SE (n = 15 rats per group). Analysis of two-way ANOVA summary: treatment effect (a): F = 8.96 at two degrees of freedom P = 0.001; time: F = 12.69, P < 0.0001 at two degrees of freedom; treatment*time: F = 23.99, P < 0.0001 at two degrees of freedom. NS not significantly different; * significantly different from UT and SH groups at P < 0.01; ** significantly different at P < 0.0001 from UT and SH groups.

As shown table 2, there were no significant differences in plasma 17 β-oestradiol (F = 2.42, P = 0.09) and progesterone (F = 0.85, P = 0.43) levels between the three groups of male after behavioural tests.

Groups	**17 β – estradiol** (ng / ml)	**Progesterone** (ng / ml)
Untreated	1.3 ± 0.1	2.1 ± 0.3
Sham	1.7 ± 0.2	1.9 ± 0.3
Nasal obstruction	1.9 ± 0.2	2.3 ± 0.2
Mean	1.6 ± 0.1^{NS}	2.1 ± 0.2^{NS}

Table 2: Plasma 17 β-oestradiol and progesterone levels at 110 days of age in male rats with and without neonatal nasal obstruction. Values are means ± SE. N = 15 rats per group. Analysis by ANOVA: NS = not significant.

4. Discussion

These results show that a short term, precocious, nasal obstruction (at 8 days postnatal for 5 days) has a profound influence on the reactivity of naïve, adult male rats to odours of same age sexually mature female rats but not to a new environment (fresh sawdust) indicating that exploratory and sniffing behaviour were normal but sexual cue behaviour was disrupted. Untreated and sham operated male rats of the same age and weight demonstrated normal behaviour to the sexual cues as well as to the new environment. Furthermore, the nasal obstructed rats had lower plasma testosterone levels but higher corticoid levels and were more reactive to stress.

NO male rats were housed with NO females until weaning and thus would have learnt their odour. Then as adults, NO males were able to recognise and prefer odours of females that had been subjected to the same "stress". The other male rats showed a specific preference for females that had not been nasally obstructed and were sexually mature. These results are in agreement with the observations of others in animals (Johnson, 1973; Gheusi & Lledo, 2005) and in humans (Martins et al., 2005).

The untreated and sham adult naïve male Wistar rats of these experiments demonstrated a clear preference for the odours of receptive (oestrus) females as compared with those of non receptive (anoestrus) females. Male rats spend more time investigating the arm of a T maze which contained the odour from a receptive female than the arm which contained the odour from a no receptive female. These results are similar to those for male Sprague-Dawley rats (Stern, 1970). Sex odour preference is very important in the rat's natural environment as it may serve as a means for selecting appropriate sexual partners. However, the significance of the odour must be learned as sexual contact is necessary for the preference to appear.

These experiments demonstrate that chemosensory information contained in urine and other secretions from conspecific females activates the HPG axis in sexually naïve adult male rats. Female odours elicited a rapid rise in plasma testosterone without affecting the other sex hormones. These experiments show in the rat an increased plasma androgen levels with exposure to female secretions as has been reported previously in hamsters (Macrides et al., 1974; Pfeiffer and Johnston, 1992; Richardson

et al., 2004). The increase in testosterone that occurs 30 min after exposure to pheromones is unlikely to be directly involved in the initial activation of reproductive behaviour, as males typically engage in the entire sequence of copulatory behaviours within minutes of being placed with a receptive female (Meek et al., 1997; Romeo et al., 1999). The rise in testosterone after exposure to chemosensory information from the female most likely serves long-term functions, e.g., altering neural or behavioural responses to females in future encounters, or in reinforcing the behaviour (Wood et al., 2004).

If a naïve laboratory rat is introduced into a novel environment, the ensuing elevation of corticosterone has been regarded as a function of the novelty of that environment (Pfister & King, 1976). The rapid rise of corticoid levels as a consequence of exposure to a novel environment that we see here has been documented also by others (Bassett et al., 1973; Hennessy & Levine, 1978; Pfister, 1979). Furthermore, an increased level of corticosterone has been shown to reduce the testosterone response (Retána-Marquez et al., 2003) and this is clearly demonstrated here with the rats of these experiments. The novel environment provoked a greatly increased corticosterone response in the obstructed rats compared to the untreated and sham rats. However, the testosterone response was attenuated in the obstructed animals. In fact it was no different from the levels before the tests indicating perhaps that there was no “sexual” stimulation. In normal rats the detection of receptive female odours is via the vomeronasal organ (Bakker et al., 1996). In contrast to the olfactory bulbs, which are the primary olfactory detection system, the vomeronasal organ does not detect long distance airborne molecules but is very sensitive to those found in close contact with urine of receptive females (O’Connell et al., 1978; Meredith & Fernandez-Fewell, 1994).

There could be other influences on sexual development that could be responsible for the results presented here. We have remarked already that in the NO group during infancy the mother spent more time licking the genital organs of males which at this time increased plasma testosterone levels (Gelhaye et al.; 2011; Padzys et al., 2011) and this has been shown to be necessary for male rat masculinisation (Moore, 1992). Furthermore, the olfactory bulbs of NO rats have been shown to be smaller than controls the day after the induction of blockade as well as at adulthood (Padzys et al., in

press). However, neither of these conditions had any reflection on the weight of the testicles suggesting that here at least development was normal (Padzys et al., in press).

It is interesting that nasal obstruction was of only a short duration and very early in the life of the rat but the effects lasted until adulthood. It did not disturb normal exploratory behaviour of the environment but uniquely aspects of sexual behaviour. This could indicate either lack of sexual maturation, slowed puberty, or an impossibility to differentiate female sex odours through decreased activation of the vomeronasal structure.

An unusual difference observed in the obstructed rats was increased levels of triglycerides and cholesterol in the plasma. These increased levels could indicate an increased basal level of stress, corroborated by the increased basal level of corticosterone. In stress there is a tendency for plasma levels of glucose to decrease and the release of glycerol and the accompanying triglycerides are needed to maintain these levels (Cahill et al., 1970). However, the rats appeared to behave normally and their exploratory behaviour of the novel sawdust was the same as the untreated and the sham rats. Increased plasma corticosterone levels support the increased plasma cholesterol levels, once again indicating increased stress-induced cholesterol levels (Bryant et al., 1988).

In conclusion, we have shown that short term nasal obstruction in 8 days old male rats can have profound effects of the capacity of these rats when adult to detect sexual odours from mature receptive females. However, it does not appear to have had a negative impact on the capacity to explore a novel environment. The long term consequence of this could influence the sexual behaviour of the males and thus their capacity to reproduce. This particular aspect remains to be investigated.

CONCLUSION et PERSPECTIVES

Chez la plupart des mammifères un développement précoce idéal dépend d'une relation stable entre la mère et l'enfant. Les soins maternels et les influences alimentaires sont cruciaux, non seulement pour régulariser la croissance du nouveau-né, mais aussi pour moduler la réponse du système neuroendocrinien au stress, et influencer certains aspects du développement cérébral.

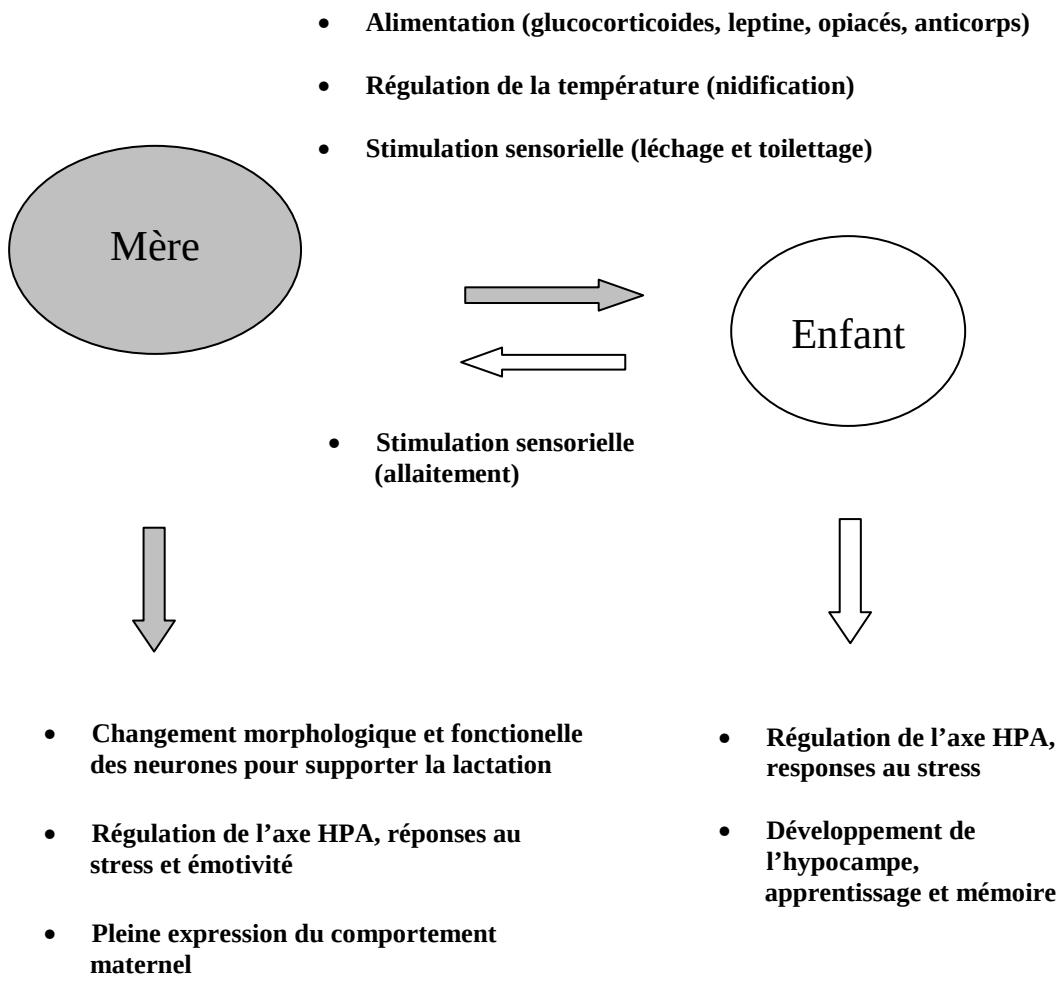

Figure 9: Représentation schématique des relations réciproques entre la mère et l'enfant (Walker et al 2004)

L'ensemble de nos résultats montre que dès le premier jour, l'obstruction nasale perturbe l'interaction mère-jeunes et le développement des jeunes. Plusieurs études (Walker *et al*, 2004 ; Morton *et al.*, 2005 ; Haynes *et al.*, 1997) montrent , que lors de la prise alimentaire, l'hormone de satiété, la leptine, contenue dans le lait maternel, constitue un facteur clé régulant aussi bien les fonctions neuroendocriniennes, que

l'homéostasie hydrique et énergétique. Les jeunes exposés à une obstruction nasale ont des difficultés à téter et donc à avaler des quantités suffisantes de leptine. Par conséquent, des études sur le rôle de la leptine pourraient être envisagées pour nous permettre de préciser l'implication de cette protéine dans le développement des jeunes Mammifères soumis à une obstruction nasale. Par ailleurs, on sait que l'administration exogène de leptine tend à réduire les effets de la privation nutritionnelle sur les axes corticotrope et thyroïdien (Ahima *et al.*, 1996). Nos résultats montrent que les perturbations dans la prise alimentaire, et l'apparition d'une déshydratation observée dès le premier jour de l'obstruction nasale, s'accompagnent d'une augmentation des taux de corticostérone, et d'une baisse de la thyroxine chez les jeunes. Ces modifications hormonales sont plus marqués chez les femelles.

D'autres données appuient aussi l'influence régulatrice inverse (fig.9), soit que la stimulation émanant du nourrisson a un effet sur l'état de la mère. Au cours de la période de lactation, les mères présentent des réponses neuroendocriniennes et comportementales moins sensibles à plusieurs types de facteurs de stress, sauf si ceux-ci menacent la vie de l'enfant. Cette capacité de «filtrer» les stimuli adéquats de ceux qui ne le sont pas, tout en s'occupant du nourrisson, peut être considérée comme une capacité d'adaptation de la relation mère-enfant. Les soins maternels sont également connus pour l'établissement de la réponse neuro-endocrine au stress, mais aussi pour la masculinisation du jeune. En effet chez le rat le léchage et le toilettage des petits, durant les deux premières semaines de sa vie, semble être nécessaire pour le développement de l'axe HPA et de la réactivité neuro-endocrine au stress. De même le léchage anogénitale du jeune mâle, est nécessaire à la masculinisation de l'aire pré-optique de l'hypothalamus. Ce qui sous entend que l'augmentation du taux de testostérone plasmatique observée chez les jeunes mâles, pourrait être liée à l'augmentation du temps de léchage de la mère durant la période d'obstruction nasale.

De nombreuses études complémentaires sont donc nécessaires afin de préciser les effets relatifs de ces différentes hormones. Par exemple, une administration de testostérone aux jeunes femelles permettrait de clarifier le rôle de cette hormone dans les différences inter-sexuelles constatées suite à l'induction de l'obstruction nasale. Le dosage des hormones hypothalamiques (CRH et TRH) et hypophysaires (ACTH et TSH) mais également de l'hormone de croissance, permettrait en outre de préciser les

mécanismes sous-jacents aux variations de concentrations en corticostérone et en hormones thyroïdiennes.

La période d'obstruction nasale provoque d'autre part la mise en place d'une respiration orale forcée, qui entraîne une adaptation rapide des muscles respiratoires se maintenant à long terme. Un examen de la dépense énergétique des animaux exposés à la respiration orale forcée, liée à l'obstruction nasale, permettrait d'analyser l'impact du travail des muscles respiratoires sur l'état physiologique des jeunes. En effet, la balance énergétique est sans doute perturbée par l'obstruction nasale précoce. Les systèmes musculaires constituent une composante importante de la dépense énergétique en termes de croissance, d'activité et de turnover protéique. Les atrophies musculaires constatées suite à l'induction de l'obstruction nasale, peuvent donc être envisagées comme un processus adaptatif visant à faire face à la demande énergétique accrue, associée à la difficulté de respirer normalement. Lors d'une situation stressante, il y a en effet compétition entre la croissance et la résistance à la perturbation pour l'utilisation des ressources énergétiques. Il est toutefois difficile, de déterminer si les atrophies observées suite à l'obstruction nasale, représentent un mécanisme adaptatif visant à économiser l'énergie ou un symptôme traduisant un état pathologique. En admettant que les modifications engendrées par l'obstruction nasale soient réversibles, celles-ci pourraient effectivement constituer un processus adaptatif facilitant la survie de l'individu. Pour pendre l'exemple de la composition en MHC des muscles oro-faciaux, ceux-ci présentent un profil en MHC adapté à la respiration buccale mais qui, du fait du retour à une respiration nasale normale, correspond en fait à un phénotype en inadéquation avec la demande environnementale. On a observé également que le développement cranio-facial chez les mâles est adapté pour permettre une respiration orale fréquente des individus. Ces adaptations ne sont plus visibles à l'âge adulte chez les mâles. C'est sans doute leur état hormonal, qui à long terme permet de rattraper les modifications observées pendant la période d'obstruction nasale. Par conséquent, une étude plus approfondie de l'action des hormones glucocorticoïdes et sexuelles (androgènes et oestrogènes) sur le développement musculo-squelettique, doit être envisagée pour mieux comprendre le dimorphisme sexuel observé à long terme dans le développement cranio-facial suite à l'obstruction nasale.

Par ailleurs, les études préliminaires ont montré que l'obstruction nasale, à court terme chez les rats mâles peut avoir des effets profonds sur la capacité de ces rats, à l'âge adulte à détecter les odeurs sexuelles femelles adultes réceptives. Toutefois, il ne semble pas avoir eu un impact négatif sur la capacité d'explorer un nouvel environnement. La conséquence à long terme de ce qui pourrait influer sur le comportement sexuel des mâles et peut être sur leur capacité à se reproduire. Cet aspect particulier reste à étudier, en particulier faire une étude neuroanatomique de l'aire préoptique de l'hypothalamus, site de contrôle de la libération des hormones gonadiques, mais également des noyaux paraventriculaire et supra-optique qui contrôlent la libération de la vasopressine et de l'ocytocine (hormone impliquée dans les relations sociales). De plus, l'étude du système limbique peut également nous permettre d'analyser les relations entre le système olfactif et le substrat anatomique de l'expression de l'émotion et du comportement.

Par ailleurs, notre étude n'a pas permis d'aborder le comportement du choix du partenaire sexuel des femelles en relation avec leur état hormonal. Il apparaît donc nécessaire également d'aborder l'étude du comportement sexuel des femelles afin de vérifier si l'obstruction nasale précoce entraîne comme chez les mâles des perturbations dans l'apparition du comportement sexuel et des modifications neuroanatomiques à court et à long terme.

En conclusion nos résultats montrent qu'une obstruction nasale réalisée du 8ème au 15ème jour postnatal, a des conséquences qui perdurent au moins jusqu'à l'âge adulte dans le cadre du développement morphologique et comportemental. Ainsi, la sous-nutrition peut perturber les fonctions neuroendocriniennes de l'adulte en modulant par exemple l'activité de l'axe corticotrope et l'axe hypothalamo-hypophyse-gonades. Par ailleurs, l'hypothyroïdisme et l'hypercorticostéronémie postnatals sont susceptibles d'avoir des conséquences permanentes sur l'organisation et le fonctionnement du système nerveux central. Ces perturbations physiologiques sont susceptibles d'intervenir dans l'apparition ou la modulation du comportement sexuel de l'individu.

L'ensemble de ces modifications observées lors de notre étude sur le rat peut contribuer à comprendre, dans une certaine mesure, certaines pathologies humaines. En effet, il serait intéressant de préciser quels facteurs agissent pour produire des changements morphologiques et physiologiques lors d'une obstruction nasale chronique chez l'enfant ou l'adulte. Ainsi nos études montrent qu'il est très important de faire un bilan de l'état

nutritionnel, du degré d'hydratation, et de mesurer régulièrement la croissance cranio-facial chez les jeunes exposés à la respiration orale forcée. En outre, l'obstruction nasale est considérée comme un facteur aggravant dans les troubles respiratoires du sommeil (Rombaux *et al*., 2005 ; Craig et al., 2008) qui chez les enfants et les adultes a un impact très négatif sur la qualité de vie, car cela entraîne entre autre une augmentation de la somnolence diurne (Udaka *et al*., 2006). Ces différentes affections correspondent à celles observées dans l'apnée obstructive du sommeil, causée par des épisodes d'obstruction des voies aériennes. Elles conduisent à une hypoxie qui modifie la structure musculaire des voies aériennes supérieures et l'expression du type de fibre de manière quelque peu similaires, à ceux que nous venons d'observer chez le rat. De même chez l'enfant comme dans les conditions pathologiques (rhinite allergique, rhinopharyngite, hypertrophie adénoïde et polypes nasaux), la suppléance orale entraîne une sécheresse buccale et à long terme des troubles de la croissance du tiers moyen de la face, avec un faciès adénoïdien classique (béance labiale et de l'articulé dentaire, palais étroit et ogival). Cela nous permet de dire que notre modèle d'obstruction nasale temporaire, peut être un modèle approprié pour la recherche sur les changements potentiels au niveau des adaptations de l'organisme face à un stress chronique. Nos résultats montrent également que la mise en place de la respiration orale chez le rat, peut avoir un effet à long terme aussi bien sur l'état physiologique (état hormonal) que sur le comportement sexuel. Cela montre l'importance de traiter rapidement l'obstruction nasale chez les nourrissons et les enfants.

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