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Sim1 function in the developing and adult brain

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Université de Montréal

Faculté des études supérieures

Cette thèse intitulée:

Sim1 fuction in the developing and adult brain

présentée par:

Chun Yang

a été évaluée par un jury composé des personnes suivantes :

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SUMMARY

Sim1, which codes for a bHLH-PAS transcription factor, is expressed in various areas of the brain, including the developing and postnatal paraventricular nucleus (PVN), a region of the hypothalamus that regulates food intake. Haploinsufficency of *Sim1* causes hyperphagia in mice and humans without any decrease of energy expenditure. Isolated hyperphagia is suggestive of PVN dysfunction. Indeed, the number of PVN cells is decreased by 24% in *Sim1*$^{+/-}$ mice, suggesting that their hyperphagia is caused by a developmental mechanism. However, the possibility that *Sim1* functions in the postnatal PVN to regulate food intake cannot be ruled out. In order to explore this hypothesis, two strategies were used.

First, in an attempt to identify a PVN specific element that could be used to modulate *Sim1* expression levels in mice, we studied the mechanism of regulation of *Sim1* gene expression. We characterized the promoter and regulatory elements in an 8.1kb 5'-sequence of *Sim1* gene. We found an AHR-ARNT/2 binding site which positively regulates promoter activity in the context of transfection experiments in Neuro-2A cells. The bHLH-PAS complex AHR-ARNT/2 can bind to this consensus site in gel shift assay. Overexpression of *Arnt, Arnt2,* or *Ahr* increased the activity of a reporter construct containing the *Sim1* promoter and the AHR-ARNT/2 binding site by 2-fold, but deletion of 4bp of its core sequence abolished this induction. Similarly, addition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand of AHR, increased the activity of the reporter construct by 1.8-fold but not that of its mutated version. Finally, we found that TCDD increased *Sim1* expression in Neuro-2A cells and in mouse kidney and hypothalamus by more than two fold. We conclude that *Sim1* expression is regulated by AHR-ARNT/2, pointing to complex regulatory interactions between bHLH-PAS proteins. However, our transgenic studies suggest that the elements required for *Sim1* expression in the PVN are out of the 5'-region was studied.

Second, we used adenoviral vectors to modulate *Sim1* expression in the postnatal PVN of wild-type mice. Unilateral stereotaxic injection into the PVN of an adenoviral vector producing a short hairpin RNA directed against *Sim1* (Ad-shRNA-*Sim1*) resulted in a significant increase of food intake. The maximal effect was about 22% which was reached on the 6th day after injection, when compared to the injection of a control virus. In contrast, injection of an adenovirus that expresses *Sim1* (Ad-*Sim1*) induced a decrease of food intake that was maximal on the 7th day after injection, reaching 20%. In order to explore the impact of more extensive changes in *Sim1* expression levels on food intake, we performed a set of bilateral injection of these viruses. Again, we found the Ad-shRNA-*Sim1* virus increased whereas Ad-*Sim1* virus decreased food intake. Although the impact of bilateral injections of these vectors into the PVN was not greater than that of unilateral injections, it further validates the results obtained from the unilateral injections. Taken together, our results strongly suggest that *Sim1* functions physiologically to regulate food intake, and support the existence of a pathway in the PVN that regulates food intake independently energy expenditures. SIM1 can be added to a growing list of PAS domain proteins that are involved in the regulation of physiological processes acting at the interface between the environment and the internal milieu.

Key Words: *Sim1*, paraventricular nucleus (PVN), bHLH-PAS protein, promoter, gene regulation, food intake, hyperphagia, adenovirus, arylhydrocarbon receptor (AHR)

RÉSUMÉ

Le facteur de transcription SIM1, qui est caractérisé par des domaines bHLH et PAS, est exprimé dans des régions spécifiques du cerveau, incluant le noyau paraventriculaire (PVN) de l'hypothalamus, une région qui participe à la régulation de la prise alimentaire. L'haploinsuffisance de *Sim1* engendre une hyperphagie chez la souris comme chez l'homme, conduisant à l'obésité. Une hyperphagie isolée est compatible avec une dysfonction du PVN. De fait, le nombre de cellules constituant le PVN est réduit de 24% chez les souris *Sim1*$^{+/-}$, suggérant que leur hyperphagie est d'origine développementale. Cependant, comme *Sim1* est exprimé de façon continue dans le PVN adulte, la possibilité que ce gène agisse le long d'une voie physiologique pour réguler la prise alimentaire n'est pas exclue. Afin d'explorer cette hypothèse, nous avons mis à profit deux approches.

Dans un premier temps, nous avons voulu utiliser la transgénèse chez la souris pour moduler l'expression de *Sim1* dans le PVN. Afin d'identifier un élément régulateur spécifiquement actif dans le PVN, nous avons étudié un fragment de 8,1 kb de séquence 5' qui contient le promoteur basal. Nous avons identifié au sein de ce fragment un site de liaison pour le complexe bHLH-PAS AHR-ARNT/2 qui contrôle positivement l'activité promotrice d'un fragment génomique de *Sim1* dans des cellules Neuro-2A. Des expériences de migration retardée sur gel ont confirmé que ce complexe lie ce site. L'expression de *Arnt*, *Arnt2* ou d'*Ahr* augmente l'activité du promoteur de *Sim1* par un facteur de deux lorsque ce site est présent en amont. De plus, l'addition de 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), un ligand de AHR, augmente l'activité du promoteur par un facteur de 1,8, mais n'a aucun effet si le site de liaison n'est pas présent. Finalement, l'administration de TCDD augmente l'expression de *Sim1* dans les cellules Neuro2A ainsi que dans le rein et l'hypothalamus de souris.

Nous concluons que l'expression de *Sim1* est régulée par le complexe AHR-ARNT/2, suggérant l'existence d'interactions régulatrices complexes entre les protéines bHLH-PAS. Par contre, des études de transgénèse indiquent que le fragment de séquence 5' ne contient pas d'éléments régulateurs spécifiquement actifs dans le PVN.

Nous avons utilisé une approche différente pour moduler l'expression de *Sim1* dans le PVN qui met à profit des vecteurs adénoviraux. L'injection stéréotaxique unilatérale d'un virus produisant un petit RNA en épingle dirigé contre *Sim1* (shRNA-*Sim1*) dans le PVN induit une augmentation de la prise alimentaire. L'effet maximal est atteint au sixième jour après l'injection : à ce stade, la prise alimentaire des souris injectées avec le virus shRNA-*Sim1* est accrue de 22% en comparaison avec celle de souris infectées avec un virus contrôle. Par contre, l'injection d'un adénovirus qui produit SIM1 (Ad-*Sim1*) induit une diminution de la prise alimentaire qui était maximale au 7e jour suivant la procédure, atteignant 20%. Afin d'évaluer l'impact de changements plus extensifs de l'expression de *Sim1* sur la prise alimentaire, nous avons effectué des injections bilatérales de ces virus. Malgré le fait que les injections unilatérales et bilatérales ont un impact similaire sur la prise alimentaire, ces expériences ont permis de confirmer l'effet de ces virus avec une deuxième série d'injections. Globalement, ces résultats suggèrent fortement que *Sim1* fonctionne le long d'une voie physiologique qui contrôle la prise alimentaire. SIM1 peut être ajouté à une liste croissante de protéines à domaines PAS qui agissent à l'interface de l'environnement et du milieu interne.

Mots clés: *Sim1*, proteine bHLH-PAS, AHR, noyau paraventriculaire (PVN), promoteur, régulation géniqueie. prise alimentaire. hyperphagie, adénovirus, réceptor arylhydrocarbon (AHR)

Table of contents

List of figures

List of abbreviations (general)

AAV, adeno-associated viral; AgRP, agouti-related protein; AHR, arylhydrocarbon receptor; AhRR, aryl hydrocarbon receptor repressor; ARNT, aryl hydrocarbon receptor nuclear translocator; BAC, bacterial artificial chromosome; BAT, brown adipose tissue; BAT, brown adipose tissue; bHLH, basic helix-loop-helix; BMAL1, brain and muscle Arnt-like protein 1; BRN2, brain 2; CART, cocaine- and amphetamine-regulated transcript; CBP, CREB binding protein; CCK, cholecystokinin; CME, central midline element; CMV, cytomegalovirus; CREB, cAMP-repsonse element binding; CRH, corticotropin-releasing hormone; CRY, cryptochrome; CTL, cytotoxic T lymphocyte; Cyp1a1, cytochrome P450, family 1, subfamily A, polypeptide 1; DRE, dioxin-responsive element; DS, Down syndrome; EMSA, electrophoretic mobility-shift assay; EPO, erythropoietin; ESTs, expressed sequence tags; GFP, green fluorescent protein; GI, gastrointestinal; GRIP1, glucocorticoid receptor interacting protein 1; GRIP1, glutamate receptor interacting protein 1; HIF, hypoxia-inducible factor; HIV-1, immunodeficiency virus type 1; HRE, hypoxia response element; Hsp90, heat shock protein 90; IPAS, inhibitory PAS domain protein; LPL, lipoprotein lipase; MC4R, melanocortin receptor 4; MSH, melanocyte stimulating hormone; NF-H, neurofilament H regulatory element; NLS, nuclear localisation sequence; NPAS, neuronal bHLH-PAS protein; NPY, neuropeptide Y; NTX, naltrexone; ODD, oxygen dependent degradation domain; OT, oxytocin; OTP, orthopedia protein; PAS, Per, ARNT, SIM homology domain; Per, period gene; POMC, pro-opiomelanocortin; PS, protease; rAAV, Recombinant AAV; RACE, rapid amplification of 5'-cDNA ends; RFP, red fluorescent protein; RSV, Rous sarcoma virus; SIM, single minded; SRC-1, steroid receptor coactivator-1; SS, somatostatin; TAD, transactivation domain; TCDD, 2,3,7,8-

tetrachlorodibenzo-p-dioxin; TIF2, transcriptional intermediary factor2; TRH, thyrotropin-releasing hormone; TSS, transcription start site; UCP, uncoupling protein; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau protein; VP, vasopressin; WAT, white adipose tissue; WT, wild-type; XAP2, hepatitis B virus X-associated protein 2; XRE, xenobiotic response element;

Neuroanatomical abbreviations

aPV, anterior periventricular nucleus; ARC, arcuate nucleus; BS: brain stem; CNS, central nervous system; DMV, dorsal motor nucleus of vagus nerve; LHA, lateral hypothalamic area; MB, mammillary body; ME, medial eminence; MTEG, mammillotegmental tract; MTH, mammillothalamic tract; NLOT, nucleus of the lateral olfactory tracr; NTS, nucleus of solitary tract; PMT, principal mammillary tract; PVN, paraventricular nucleus; rNST, rostral nucleus of solitary tract; SC: spinal cord; SCN, suprachiasmatics nucleus; SON, supraoptic nucleus; ZLI, zona limitans intrathalamica;

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DEDICATION:

To my parents, for teaching me to be a nice person.

To my husband, for his profound support and love.

To my daughter, for her understanding.

CHAPTER I: GENERAL INTRODUCTION

Studies of development pathways, cell growth and cell death have in part become studies of transcriptional regulators. Efforts to identify the nature of these regulators have revealed families of proteins that are characterized by highly conserved DNA binding, dimerization, transactivation and repression motifs. An emerging family of transcriptional regulators is characterized by the presence of basic-helix-loop-helix (bHLH) and PAS domain. These proteins control a variety of developmental and physiological processes including neural development, toxin metabolism, respond to hypoxia, circadian rhythms, tracheal formation, and hormone receptor function.

1. The bHLH-PAS proteins

The bHLH-PAS proteins usually function as dimeric DNA-binding protein complexes. Most bHLH-PAS family members can be grouped into two classes (fig. 1; Kewley et al., 2004). Class I bHLH/PAS factors neither homodimerize nor heterodimerize with other class I factors. This group includes the aryl hydrocarbon receptor (AHR), aryl hydrocarbon receptor repressor (AHRR), the hypoxia inducible factors (HIF; HIF-1α, HIF-2α, and HIF-3α), CLOCK, neuronal bHLH-PAS protein (NPAS2), and single minded proteins (SIM1 and SIM2) (Ema et al., 1996; Lindebro et al., 1995; Wang et al., 1995). They must dimerize with a class II bHLH/PAS factor to form active transcription factor complexes. The best characterized class II protein is the aryl hydrocarbon receptor translocator (ARNT) (Hoffman et al., 1991), which is ubiquitously expressed in mesodermal derived tissues. Other members of this class include ARNT2, which is predominantly expressed in the central nervous system (CNS), and the circadian rhythm proteins, brain and muscle ARNT-like protein (BMAL1/MOP3 and BMAL2/MOP9), whose expression is more restricted (Hirose et al., 1996; Okano et al., 2001). Typically, a class II protein can bind different class I proteins. The combinatorial and interactive properties of bHLH-PAS proteins

provide a variety of potential mechanisms to control their function as transcriptional regulators, which may help explain their widespread use in complex biological events.
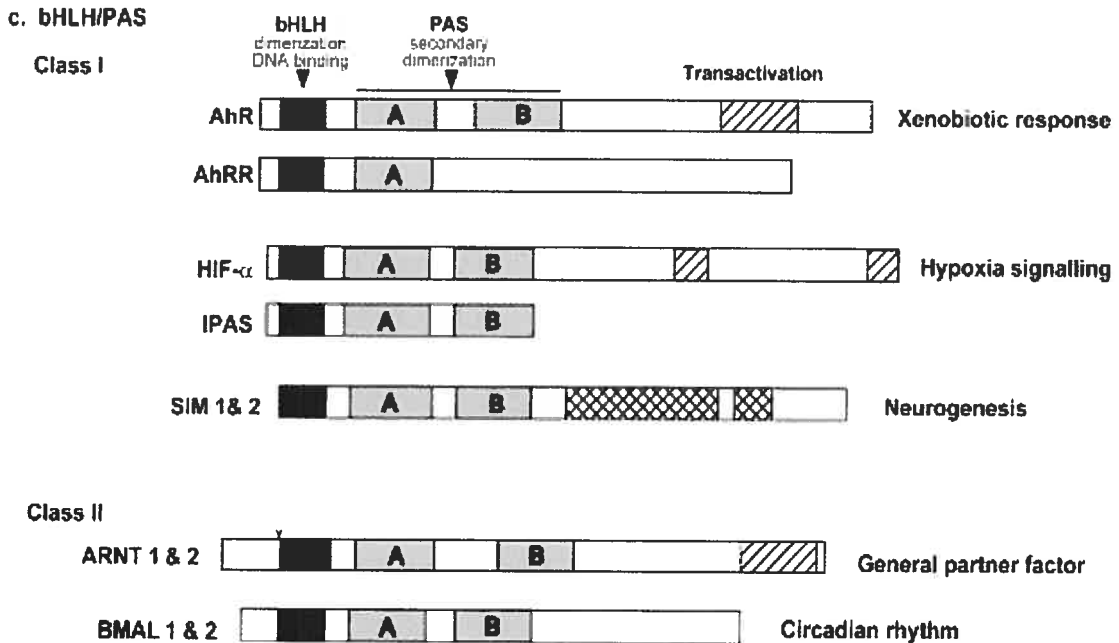
Figure 1. Schematic representation of the domain structure of some bHLH-PAS family members (Kewley et al., 2004).

A third class of bHLH-PAS protein is characterized by the ability to function as coactivators. Most notably, three bHLH-PAS proteins, steroid receptor coactivator-1 (SRC-1), murine transcriptional intermediary factor 2 (TIF2) and human glucocorticoid receptor interacting protein 1 (GRIP1), have been shown to act as coactivators for members of the nuclear receptor superfamily (Glass et al., 1997). These class III proteins have been shown to mediate the interaction between nuclear receptors and transcriptional activator/integrators such as CBP/p300 allowing full transcriptional activity of the former (Yao et al., 1996; Onate et al., 1998). It appears that neither the bHLH nor PAS domains of these proteins are required for coactivator activity (Heery et al., 1997). Most interestingly, some recent experiments suggest that bHLH-PAS proteins AHR

and ARNT also interact with other nuclear receptors, such as estrogen receptors, and may also function as coactivators (Ohtake et al., 2003; Brunnberg et al., 2003). These observations raise the possibility that proteins of class I and II may have more than one cellular role.

1.1. Highly conserved structures of bHLH-PAS proteins

The primary structure of bHLH-PAS proteins is remarkably conserved (fig.1). The bHLH domain is located near the amino terminus. The basic region binds an E box sequence that shows the consensus sequence NACGTG. Typically, each component of the dimmer binds half of the E box element sequence, whereas class II protein usually binds the GTG sequence (Swanson et al., 1995; Huffman et al., 2001). The HLH domain promotes dimerization. These residues are followed closely by the PAS domain, named for the first three proteins identified with this motif: the *Drosophila* Period (Per), human ARNT, and *Drosophila* Single-minded (sim) (Nambu et al., 1996). The PAS domain found in bHLH-PAS proteins is ~260-310 amino acids long; it is subdivided into two well-conserved regions, PAS-A and PAS-B, which are separated by a poorly conserved region. Within both the A and B regions lies a copy of a 44-amino acid repeat referred to as the PAS repeat (Crews et al., 1988; Nambu et al., 1996). This domain has been identified in proteins throughout the animal kingdom, as well as in bacteria, fungi and yeast. PAS domain is used for dimerization between PAS proteins (Huang et al., 1993), small molecule binding (Shimizu et al., 2000), and interactions with non-PAS proteins (Zelzer et al., 1997; Coumailleau et al., 1995; Gekakis et al., 1995). PAS protein can sense, directly or indirectly, a variety of signals including the redox status of the cell, glucose levels, oxygen and carbon monoxide levels and the presence of toxic compounds. Moreover, bacteria photoactive yellow protein containing PAS-like domains can detect light (Crews & Fan, 1999; Taylor & Zhulin, 1999). Finally, although the carboxy-terminal regions of bHLH-PAS proteins are not well conserved, they typically harbour transcriptional activation

or repression domains (Franks & Crews, 1994; Jain et al., 1994; Li et al., 1994; Moffett et al., 1997).

1.2. Regulation of physiological processes by bHLH-PAS proteins

1.2.1. AHR mediating xenobiotic metabolism

Dioxin (2,3,7,8-Tetrachlorodibenzo-p-dioxin, TCDD) and structurally related halogenated aromatic hydrocarbons are members of a class of environmental pollutants. It is formed during combustion of waste. Due to its lipophilicity and resistance to physical and biological breakdown, it persists and accumulates in the environment and the food chain. A body of evidence has suggested a wide spectrum of toxic effects of TCDD on growth, reproduction, the immune system and the endocrine system in laboratory animals and humans (Pohjanvirta et al., 1994; Unkila et al., 1995). Acute administration of a sub-lethal dose of TCDD to adult animals induces a variety of severe toxic effects, including anorexia, decrease of body weight, hypothermia and reduced locomotor activity (Christian et al., 1986; Tuomisto et al., 1995).

On a molecular level, responses to TCDD and related chemicals are mediated through the AHR. The AHR (also known as the Dioxin receptor) was one of the first bHLH-PAS factors to be cloned. It is activated by dioxin and related environmental pollutants to regulate genes encoding xenobiotic metabolising enzymes and mediates the severe toxicity associated with these compounds (Shimizu et al., 2000). In its latent (non-DNA binding) state, AHR is found in the cytoplasm, stably associated with at least three molecules, the 90 kDa molecular chaperone heat shock protein 90 (Hsp90), p23 and hepatitis B virus X-associated protein (XAP2/AIP/Ara9) (Kazlauskas et al., 1999; Meyer et al., 1998; Ma & whitlock, 1997). Following ligand binding, the AHR/Hsp90 complex translocates to the nucleus where Hsp90 is exchanged for the dimerizing partner ARNT (Lee & Whitelaw, 1999; Pollenz et al., 1994). The nuclear localised, ligand-bound AHR-ARNT heterodimer promotes transcription by binding xenobiotic response

elements (XREs) found upstream of TCDD-responsive genes, such as those coding for the xenobiotic metabolising enzymes cytochrome P4501A1 and the glutathione *S*-transferase Ya subunit (Poellinger, 1995). One of AHR target genes is another bHLH-PAS member, AH receptor repressor (AHRR), the product of which acts as a negative regulator competing with AHR for binding to ARNT (Mimura et al., 1999; Karchner et al., 2002). (fig. 2).
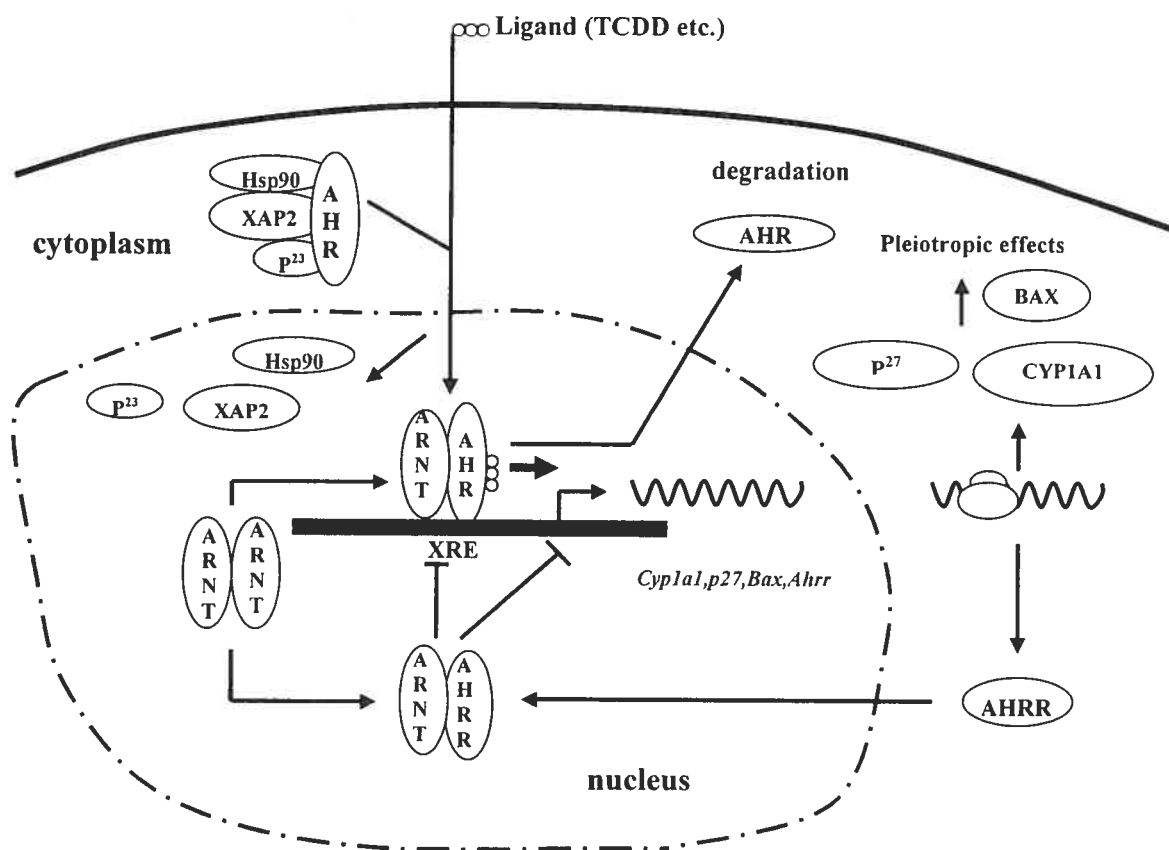
Figure 2. Mechanisms of transcriptional activation by AHR and of negative feedback regulation of AHR by AHRR (modified from Mimura & Fujii-kuriyama, 2003). Upon binding to a ligand, cytoplasm located AHR is translocated to the nucleus where it dimerizes with ARNT and activates transcription of target genes, including *Ahrr*. In turn, the latter will compete with AHR for binding to ARNT.

AhR is expressed in a variety of tissues during development and in the adult rodent (Carver et al., 1994). The phenotype resulting from gene knockout experiments in mice is consistent with a developmental role for the *AhR*. *AhR*^{−/−} mice have been generated by three research groups with a common phenotype characterized by smaller livers and decreased body weight (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Schmidt & Bradfield, 1996). The reduction in liver size in *AhR* null animals correlates with reduced hepatocyte size and a high degree of portosystemic shunting, due to defective vascularisation (Lahvis et al., 2000). Concerning xenobiotic metabolism, the lack of *AhR* abolished the inducible expression of *CYP1A1* and *1A2* genes in response to TCDD (Mimura et al., 1997; Shimizu et al., 2000). These data suggest that *AhR* has an important role in mouse development, and confirmed its requirement for dioxin metabolism (Gu et al., 2000).

The expression of *AhR* and *Arnt* has been demonstrated in the hypothalamus of the rat (Huang et al., 2000; Huang et al., 2003) along with a high expression of *Arnt2*, which is a dimerization partner of AHR in the central nervous system (Petersen et al., 2000). In the rat, strong expression of *Arnt2* has been found in the supraoptic nucleus (SON) and in the paraventricular nucleus (PVN), and low or moderate levels in most hypothalamic regions. Altogether, *AhR, Arnt* and *Arnt2* are expressed in several regions that play a role in the regulation of feeding behavior and body weight (Petersen et al., 2000). TCDD-induced effects have been well characterized in the liver, whereas less is known about the effects of TCDD on the central nervous system. TCDD penetrates poorly into the rat brain and the concentrations found in the latter tissue are far lower than in liver or adipose tissue (Pohjanvirta et al., 1990). However, sensitive biochemical effects such as enzyme induction, and changes in neurotransmitter concentrations or turnover rates are seen in the brain after TCDD exposure (Unkila et al., 1995).

Furthermore, after repeated exposure, TCDD induces more extensive oxidative damage in brain tissues than in liver (Hassoun et al., 2000). All these finding suggest that TCDD may exert a direct effect on the CNS. Although the genes underlying the adaptive response to dioxins have been well characterized, the molecular mechanism underlying most aspects of the AHR-mediated toxic response is currently unknown.

Several groups have reported the identification of endogenous ligands for AHR (Bonnesen et al., 2001; Schaldach et al., 1999; Sinal and Bend, 1997; Heath-Pagliuso et al., 1998). The strongest example is the indigo-related compounds (Adachi et al., 2001) However, because these compounds were isolated from human urine, the question of whether they represent excretion products from an exogenous source or were generated from endogenous compounds remains unanswered. Similarly, AHR binding molecules such as lipoxin A, bilirubin-related compounds, and tryptophan-related compounds (indole and tryptamine), are certainly endogenous but whether they are the true physiological ligands for AHR has not yet been resolved. The DeLuca group claimed the successful identification of an endogenous ligand for AHR with a structure deduced as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE). Recognition of this endogenous ligand is conserved from fish to human (Song et al., 2002). With the availability of this ligand, physiological functions of the AHR can be directly investigated.

1.2.2. Regulation of hypoxia responsiveness by HIF

The ability to maintain O_2 homeostasis is essential for survival of mammals. The hyperoxic state, or high O_2 tension, can result in the generation of reactive oxygen intermediates and potentially lethal damage to membranes and DNA. The hypoxic state, or low O_2 tension, can result in levels of ATP insufficient to maintain essential cellular functions. The hypoxic state occurs in a number of medical conditions, such as cancer and ischemia, inspiring research into

understanding the cellular mechanisms for detecting and responding to low levels of oxygen.

Responses to hypoxia are mediated by three bHLH-PAS proteins, HIF-1α, HIF-2α (also known as Endothelial PAS domain protein 1), and HIF-3α (Ema et al., 1997; Gu et al., 1998). HIF-1α and HIF-2α (termed collectively HIF-α subunits) share 48% amino acid sequence identity and both contain an oxygen dependent degradation domain (ODD) located in the carboxy-terminal region, as well as N-terminal and C-terminal transactivation domains (N-TAD and C-TAD). As predicted from identity studies, both HIF-1α and HIF-2α show similar mechanisms of regulation (O'Rourke et al., 1999). At normoxia (20% O_2), HIF-α is continuously synthesized and rapidly degraded by the ubiquitin-proteasome system (Huang et al., 1998). Under low oxygen tension (hypoxia), the oxygen-dependent prolyl hydroxylase is inactivated. This enzyme hydroxylates a specific proline residue within a highly conserved region of the HIF-α's internal ODD, which is necessary and sufficient for forming a complex that activates the ubiquitin-E3 ligase. HIF-α protein accumulates and dimerises with ARNT (also known as HIF-1β) in the nucleus through the bHLH and PAS domains to form a functional DNA binding complex. The HIF-α-ARNT dimer binds to hypoxia response elements (HREs) in the enhancer region of target genes, recruits the transcriptional coactivator CBP/p300 and initiates transcription. The known target genes of the HIF-1 complex are involved in an adaptative response to hypoxia leading to increased glycolysis, erythropoiesis and angiogenesis. They include erythropoietin, which induces production of red blood cells, and vascular endothelial growth factor (VEGF), a key regulator of blood vessel growth (Bunn & Poyton, 1996; Guillemin & Krasnow, 1997).

Most human tissues express both *Hif-1α* and *Hif-2α* mRNA. Mice deficient in *Hif-1α* die *in utero* by embryonic day 10.5, with embryos exhibiting poor vascularisation (Ryan et al., 1998). Three independent *Hif-2α* knockout mice have been generated, pointing to at least three distinct roles. The relative importance of

these functions, however, may depend on strain background. One study demonstrates the need for *Hif-2α* in the regulation of catecholamine homeostasis (Tian et al., 1998). A second *Hif-2α* knockout mouse displayed varying degrees of vascular disorganisation and haemorrhage, indicating that *Hif-2α* is required for the control of vascular remodelling (Compernolle et al., 2002). In contrast, a third *Hif-2α* deficient mouse was found to die neonatally of respiratory distress syndrome due to impaired lung maturation (Peng et al., 2000).

A third HIF protein is also able to dimerize with ARNT and bind to the HRE sequence *in vitro*. Among the numerous splice variants of the *Hif-3α* gene locus, the best characterized is the inhibitory PAS domain protein (IPAS) (fig.1). IPAS does not have the C-terminal region containing the transactivation domain and has been reported to inhibit HIF-1α through a dominant negative mechanism (Maynard et al., 2003; Makino et al., 2001). Little is known about the *in vivo* role of HIF-3α and the IPAS splice variant.

Some evidence also suggested that HIF-α could signal through heterologous interactions with non-PAS containing proteins. HIF-1α has been shown to be involved in the stabilization of p53 and may play a role in hypoxia-induced apoptosis (An et al., 1998). Another experiment has suggested that the interaction between HIF-1α and the VHL protein, the product of the von Hippel-Lindau (VHL) tumor suppressor gene, is necessary for the oxygen-dependent degradation of HIF-α subunits (Maxwell et al., 1999). Such a relationship may explain the rich vascularization of tumors of VHL patients because α-class HIF subunits would be constitutively up-regulated in the absence of the VHL protein (Kaelin & Maher, 1998).

1.2.3. Regulation of circadian rhythm by bHLH-PAS proteins

Biological clocks help entrain an organism's activity to changes in daily and seasonal environment. Vertebrates and invertebrates employ orthologs of a number of PAS proteins, including PER, CLOCK, and BMAL1/MOP3, to control

this important biological process (Dunlap, 1999). Like their *Drosophila* homologue, mRNA levels of the mammalian *Per* respond to light and phase shifts in a circadian manner in the suprachiasmatic nucleus of the hypothalamus, the site of the master circadian oscillator in mammals. Two bHLH-PAS transcription factors, CLOCK and BMAL1, form a heterodimer and bind to the response element sequences CACGTGACC, termed M34RE (or a circadian responsive E-box), which is present in the enhancer/promoter regions of circadian-regulated genes such as *Period (Per)* and *Cryptochrome (Cry)*. As a result, the CLOCK-BMAL1 heterodimer positively regulates the levels of circadian responsive gene products (Gekakis et al., 1995). In return, PER and CRY negatively regulates the CLOCK-BMAL1 complex, either by binding to one member of the complex and disrupting its function or by indirectly influencing the signalling of the CLOCK-BMAL1 complex through interactions with the basal transcriptional machinery (Darlington et al., 1998; Lee et al., 1999; Kume et al., 1999). Because of the feedback inhibition mechanism, and a delay between transcription and function interference in the CLOCK-BMAL1 complex, an oscillation occurs and is maintained.

Neuronal bHLH-PAS Protein (NPAS2) is mainly expressed in the forebrain of mammals and is also implicated in controlling circadian rhythm (Hogenesch et al., 1998; Reick et al., 2001). The NPAS2 protein is very similar in sequence to CLOCK (Zhou et al., 1997) and can engage in a similar heterodimeric partnership with the BMAL1 protein (Rutter et al., 2001). Like the CLOCK-BMAL1 heterodimers, NPAS2-BMAL1 can activate expression of the *Per* and *Cry* (Reick et al., 2001; Sancar, 2004). The products of these genes in turn inactivate NPAS2:BMAL1 or CLOCK-BMAL1, providing feedback for circadian cycling. The main difference between NPAS2 and CLOCK appears to be in their tissue distribution. NPAS2 is abundant in the somatosensory cerebral cortex but absent from the suprachiasmatic nucleus (SCN), whereas CLOCK is found in many brain regions but is most abundant in the SCN. Mice deficient in *Clock* exhibit

abnormally long circadian cycle length of locomotor activity, with a loss of rhythmicity after a few weeks in constant conditions (Vitaterna et al., 1994). Mice without *NPAS2* show abnormal sleep patterns and fast rather than eat when food is provided only during daylight and as a result loose weight and become sick (Dudley et al., 2003). These findings demonstrate that NPAS2 of the forebrain oscillator cooperates with CLOCK to influence circadian behavior. Very interestingly, new evidence shows that *Clock* mutant mice are hyperphagic and obese (Tureck et al., 2005). It is not clear whether this phenotype results from disruption of circadian rhythm or is related to a second role of CLOCK in the hypothalamus (Manev & Uz, 2006).

The NPAS2 protein has two PAS domains, PAS-A and PAS-B, both of which bind heme (Dioum et al., 2002). Heterodimerization of NPAS2 with a partner such as BMAL1 is required for recognition of specific binding sites in enhancer sequences in DNA (Rutter et al., 2001). BMAL1 on its own forms unproductive homodimers that cannot activate transcription, but when NADPH-to-NADP ratios are high, NPAS2 can displace a BMAL1 molecule from its homodimeric partner to form a productive NPAS2-BMAL1 heterodimer (Rutter et al., 2001). Therefore, the redox status of the cells influences the activity of NPAS2. Furthermore, CO molecules produced by heme oxygenase-2 also modulate DNA binding of NPAS2-BMAL1 in the presence of a heme bound to the PAS domains. At low micromolar levels of CO, heme forms a complex with CO, resulting in inhibition of DNA binding of NPAS2 (Dioum et al., 2002). Thus CO is a likely candidate for the native signal of NPAS2, which provides another example of the modulation of a bHLH-PAS protein by small molecules.

1.2.4. ARNT and ARNT2: functional partners of bHLH-PAS proteins

bHLH-PAS proteins form dimeric transcription factors to mediate diverse biological functions. ARNT plays a central role as a common heterodimerization partner (fig. 3). Two genes encode different forms of ARNT in rodents: *Arnt*,

which is widely expressed in mesodermal derived tissues, but weakly expressed in the brain. In contrast, *Arnt2* is expressed strongly in the brain and in the kidney, but weakly in other tissues. Therefore, the expression pattern of *Arnt* and *Arnt2* appears somewhat complementary. ARNT was isolated as a factor required for the nuclear translocation of AHR from the cytoplasm in response to xenobiotics. ARNT is a protein that is constitutively located in the nucleus (Hoffman et al., 1991). ARNT contains a strong C-terminal transactivation domain which is functionally distinct from the DNA binding and heterodimerisation domains (Whitelaw et al., 1994). Transactivation by ARNT is mediated through an interaction with the CBP/p300 coactivator (Kobayashi et al., 1997).

The generation of *Arnt$^{-/-}$* embryonic stem cells and *Arnt$^{-/-}$* mice demonstrates the importance of HIF-1α-ARNT interactions for response to hypoxia, and the importance of ARNT during development (Kozak et al., 1997; Maltepe et al., 1997). *Arnt$^{-/-}$* embryonic stem cells fail to activate genes that are normally induced by low oxygen tension or decrease of glucose concentration, indicating a crucial role for ARNT in the response to hypoxia and hypoglycaemia. Moreover, mouse embryos generated from these cells are not viable beyond embryonic day 10.5. The primary cause of their lethality can be attributed to defects in vascularisation of the placenta and/or developing yolk sac and solid tissue. A similar phenotype was reported in mice deficient in *VEGF*, an HIF-1α-ARNT target gene (Carmeliet et al., 1996; Ferrara et al., 1996). It is thought that the increase in tissue mass during organogenesis generates a local hypoxic environment with consequent activation of the HIF-1α-ARNT complex, leading to increased expression of genes that promote vascularisation of the developing yolk sac and solid tissues. This model is supported by findings that the HIF-1α complex is activated in hypoxic regions of solid tumours, initiating angiogenesis and supporting tumour growth (Maxwell et al., 1997).

Like ARNT protein, ARNT2 also forms complexes with SIMs, HIF-1 and AHR proteins (Michaud et al., 2000; Maltepe et al., 2000; Gu et al., 1998). In contrast to *Arnt* mutants, *Arnt2*$^{-/-}$ embryos survive until birth without gross defects but die perinatally and exhibit subtle hypothalamic abnormalities (see below) (Keith et al., 2001; Hosoya et al., 2001; Michaud et al., 2000). In addition, *Arnt2*$^{-/-}$ neurons display decreased hypoxic induction of HIF-1 target genes in culture, demonstrating that HIF-1α-ARNT2 complexes regulate oxygen-responsive genes. A strong genetic interaction between *Arnt* and *Arnt2* was revealed. Embryos with fewer than two wild-type alleles of either *Arnt* or *Arnt2*, in any combination, are absent or severely under-represented at embryonic day 8.5 (E8.5), indicating that either gene can fulfil essential functions in a dose-dependent manner before E8.5. These finding suggests that *Arnt* and *Arnt2* have both unique and overlapping essential functions in embryonic development (Keith et al., 2001).
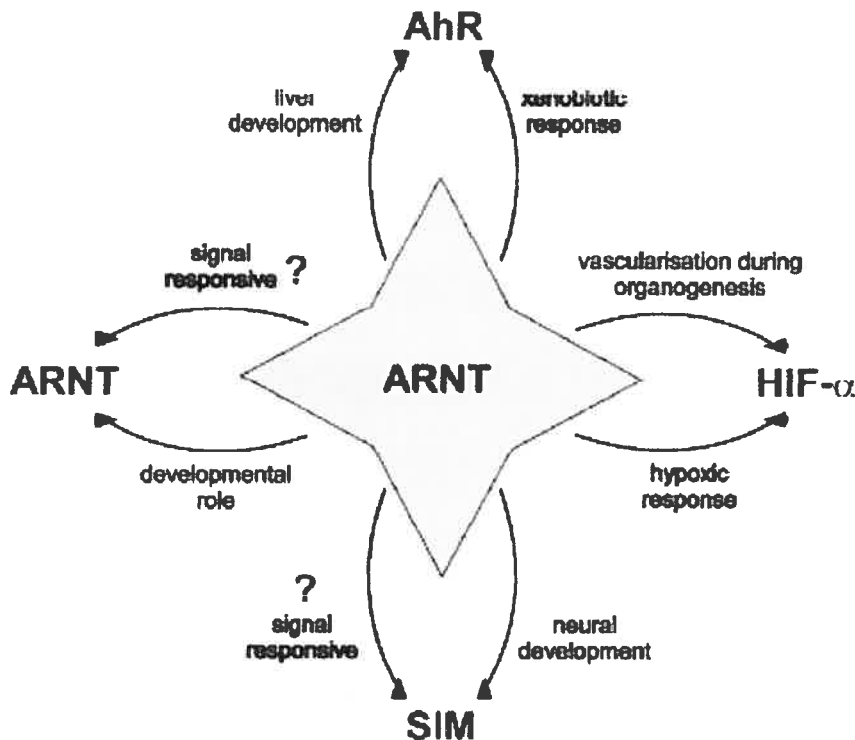
Figure 3. ARNT forms both homodimers and heterodimers with AHR, HIF-α and SIM which play roles both during mammalian development and in response to

environmental stimuli in mammals. Symbol '?' indicates where these roles have yet to be characterized (Kewley et al., 2004).

1.2.5. Crosstalk between bHLH-PAS protein-mediated signalling pathways

The fact that bHLH-PAS proteins could be involved in more than one cellular pathway raises the possibility that signalling through one pathway could influence the responsiveness to another (Schmidt & Bradfield, 1996). Such a situation could arise when parallel pathways within the same cell share a limiting common partner, such as ARNT. In support of this idea, it has been shown that AHR and HIF-1α compete for binding to ARNT in vitro decreasing these signalling pathways (Chan et al., 1999; Gassmann et al., 1997). Although the simplest explanation of these data is that ARNT is a limiting factor, these experiments do not formally exclude other interpretations, such as the importance of other shared and limiting factors are important. In this regard, the significance of limiting heterologous factors is suggested by the interference between the dioxin and the progesterone signalling pathway, and between dioxin and oestrogen receptor signalling pathway (Kuil et al., 1998; Ohtake et al., 2003). Adding to the potential complexity of the cross-talk concept is the observation that certain target genes may be regulated by two bHLH-PAS heterodimers competing for the same binding sequence. For example, SIM1-ARNT and HIF-ARNT dimers can compete for binding to the HRE in the *Epo* enhancer (Woods & Whitelaw, 2002). Recently, a novel bHLH-PAS factor, NXF has been shown to compete with SIM2 for binding to target genes (Ooe et al., 2004). It has been demonstrated that reciprocal inhibitory crosstalk between the hypoxia and dioxin signal transduction pathways can occur within mammalian cells. For instance, the promoter of human *Epo* gene is influenced by both the classical HREs in its 3' regions, as well as a number of degenerated XREs immediately upstream of its promoter (Chan et al., 1999). In addition to these parallel interactions, bHLH-PAS proteins also can interact in a hierarchical manner. For example, *Drosophila* sim positively and

directly regulates its own expression through interaction with TANGO, a homolog of ARNT (Michaud et al., 2001). Such complex interplay between bHLH-PAS proteins may enable the cell to adapt its response to multiple environmental and developmental signals, and physiological processes could influence each other through interactions between bHLH-PAS proteins.

2. SIM proteins

2.1. *Drosophila* sim, a regulator of midline development

sim functions as a master regulator of the development of the midline of the CNS in *Drosophila,* acting upstream of all known developmental processes in this region of the embryos (Crews et al., 1988; Nambu et al., 1993). sim protein is expressed in the midline cells throughout neurogenesis as well as in differentiated midline neurons and glia. Null mutants of s*im* show a complete absence of midline cell development: precursor cells fail to divide, and do not undergo differentiation into neurons and glia. In contrast, when ectopically expressed, *sim* can convert the lateral CNS to acquire the identity of the CNS midline (Thomas et al., 1988; Nambu et al., 1991). sim contains a C-terminal domain, acting as a transcriptional activator upon dimerization with Tango (Sonnenfeld et al., 1997). Tango is localized to the cytoplasm in the absence of a dimerizing bHLH-PAS protein (Ward et al., 1998). When sim is present, it forms a dimer with Tango and enters the nucleus (Shue & Kohtz, 1994). The sim-Tango complex binds a midline enhancer element (CME, ACGTG core sequence) that resides on target genes. Four *sim* target genes, *breathless* (*btl*), *sim, slit,* and *Toll* (*Tl*), have been characterized. Each of these genes is regulated differently: *Tl* is expressed in midline precursor cells; *sim* is an autoregulatory target; *slit* is expressed in differentiated midline glia cells; and *btl* is expressed in both midline and tracheal cells (Nambu et al., 1990; Crews, 1998).

2.2. Sequence similarity of the mammalian SIM proteins

Two murine homologues of *sim, Sim1* and *Sim2*, were isolated from a mouse genomic library at low stringency using a probe corresponding to the sim bHLH domain (Fan et al., 1996). The bHLH-PAS domains of the *Drosophila sim* gene product are highly conserved in the mouse (fig. 4). Overall, SIM1 and SIM2 share 69% and 65% amino acid identity with that of their *Drosophila* counterpart. Most remarkably, the amino acid sequences of SIM1 and SIM2 bHLH-PAS domain show a striking identity (86% identify). However, no significant identity is seen at the C-terminus of the three proteins (Fan et al., 1996; Ema et al., 1996).

sim

65%   69%

SIM2   SIM1

86%
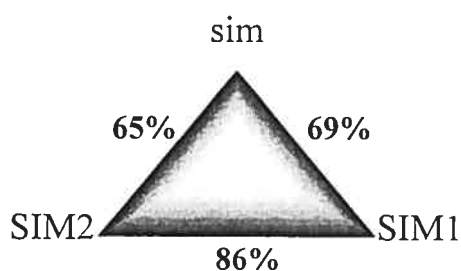
Figure 4. Amino acid identity of three SIM proteins.

2.3. Transcriptional properties of mammalian SIM proteins

Murine *Sim* homologs share a number of functional similarities with *Drosophila sim*. SIM1 and SIM2 are nuclear proteins, which bind the molecular chaperone hsp90 and undergo cytoplasmic/nuclear shuttling before forming active heterodimers with ARNT or ARNT2 (Ema et al., 1996). In cell transfection assays, the heterodimer SIM1-ARNT/2 transactivates reporter constructs containing the CME via the ARNT/2 C-terminus (Moffett & Pelletier, 2000). By itself, SIM1 has neither activation nor repression activity. Drosophila sim functions as a transactivator in the same assay, whereas SIM2 represses transcription by quenching ARNT/2 transactivation (Moffett et al., 1997; Moffett and Pelletier, 2000). The difference of activity between the SIM proteins is correlated with the observation that the C-termini of the three proteins have no

significant amino acid homology. In addition to binding to classic CME, SIM proteins are capable of binding mammalian HRE sequences in combination with ARNT (Woods & Whitelaw, 2002). SIM1-ARNT heterodimer has been shown to activate transcription form *erythropoietin* hypoxic enhancer via the transcription activation domain (TAD) of ARNT. In contrast, SIM2-ARNT heterodimers do not transactivate this construct, due to a repressive activity present within the C-terminus of SIM2. Moreover, it remains unclear whether the SIM proteins are ligand-responsive or exhibit their effects through their spatially and temporally restricted expression patterns. While no direct target genes have as yet been identified for *Sim1 or Sim2*, expression and knockout studies have given us insight into their possible roles.

2.4. Expression patterns of mammalian *Sim* genes in the brain

In situ hybridization of mouse embryos revealed that *Sim1* and *Sim2* expression in the brain is restricted. *Sim1* is expressed in regions immediately adjacent to the ventral midline of the spinal cord, mesencephalon and hypothalamus. Moreover, *Sim1* is expressed in the zona limitans of the thalamus and in the nucleus of the lateral olfactory tract (NLOT) of the amygdala (Holder et al., 2004). *Sim2* is also expressed in the ventral mesencephalon and hypothalamus as well as in the zona limitans (Ema et al., 1996, Fan et al., 1996; Yamaki et al., 1996). In the hypothalamus, *Sim1* is expressed at the highest levels in the paraventricular nucleus (PVN), in the supraoptic nucleus (SON), in scattered cells located ventromedially as well as in the mammillary body (MB), whereas *Sim2* is expressed in the PVN and in the MB, but not in the SON (Wang & Lufkin, 2000; Goshu et al., 2002) (fig. 5). *Sim1* is continuously expressed in these hypothalamus and amygdala domains after birth, whereas *Sim2* expression in the brain dramatically decreases after E16.5. In the periphery, expression of *Sim2* is found in facial and trunk cartilage, as well as trunk muscles (Fan et al., 1996). *Sim1* and

Sim2 are expressed in the kidney during development as well as in adulthood (Ema et al., 1996).
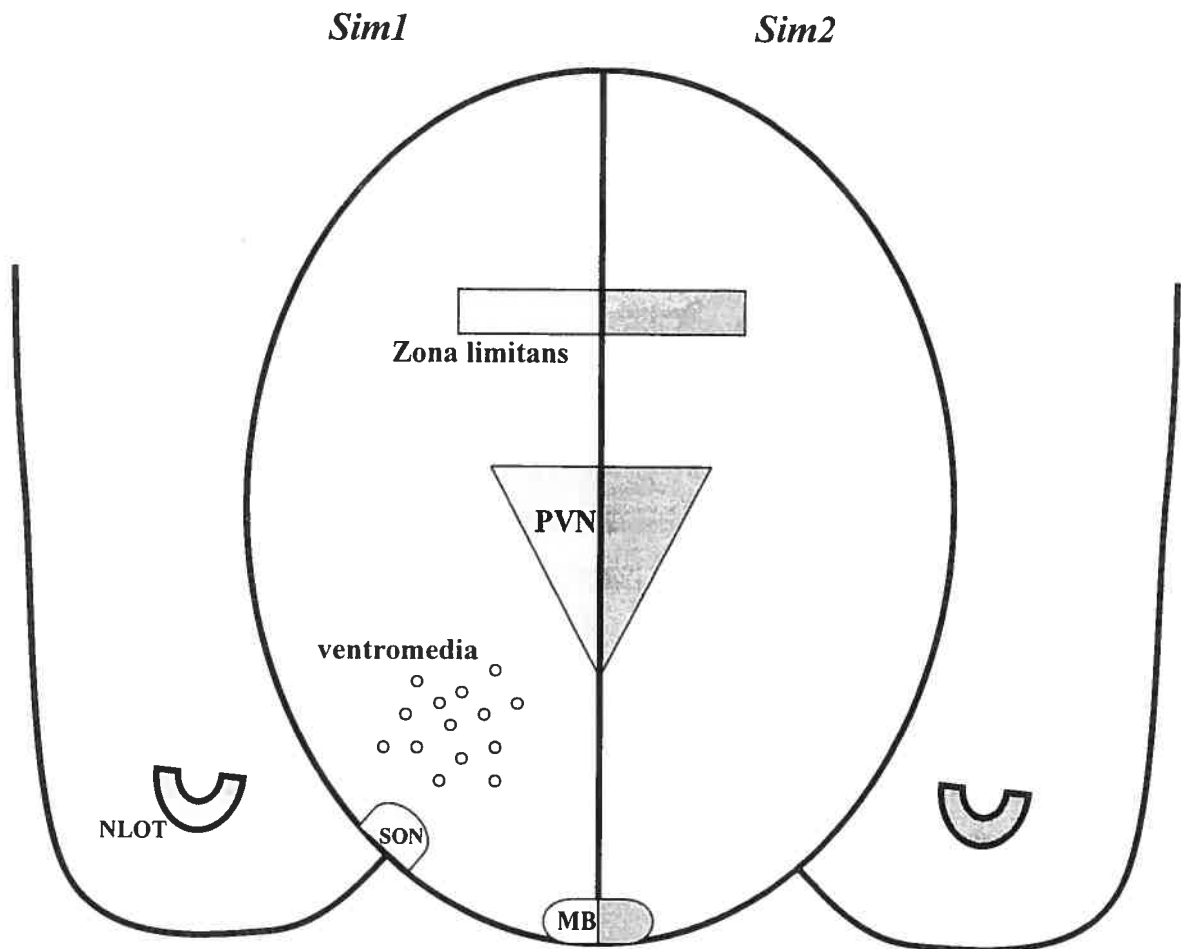
Figure 5. Expression pattern of *Sim1* and *Sim2* in the developing brain. Left side represents the of *Sim1* domains of expression, and the right side represents the *Sim2 domains of* expression. NLOT: nucleus of the lateral olfactory tract.

3. The paraventricular nucleus of the hypothalamus

The hypothalamus is located in the most medial aspect of the brain, along the walls and floor of the 3rd ventricle (Alam et al., 1995; Markakis, 2002; Sherin, et al., 1996). The hypothalamus is an evolutionary ancient integrator of homeostasis that regulates basic processes such as food and water intake, energy expenditure, the response to stress, blood pressure and reproduction. Structurally, the hypothalamus is composed of a dozen small nuclei interspaced between less defined regions. A great body of work involving physiological and genetic studies has assigned specific functions to each of these nuclei and regions (See fig. 6, Caqueret et al., 2005.). One nucleus, the PVN, is of special interest since it has been shown to participate in several physiological processes including the control of energy balance, stress responsiveness, thermogenesis and of blood pressure.

3.1. Structure of paraventricular nucleus

The PVN of the anterior hypothalamus contains several subregions which harbour distinct neuronal cell types. These neurons can be classified in at least three groups based on their axonal projections (Swanson & Sawchenko, 1983; Sawchenko et al., 1992):

1.) Magnocellular neurons project their axons to the posterior pituitary where they secrete two hormones, oxytocin (OT) and vasopressin (VP), directly into the general circulation. OT promotes lactation and labor whereas VP maintains blood pressure. The SON, which is derived from the PVN, is mainly composed of magnocellular neurons that produce OT and VP neurons. The expression of OT and VP in magnocellular neurons is mutually exclusive, defining two distinct cell types (fig. 6).

2.) Hypophysiotropic parvocellular neurons project their axons to the medial eminence where they release several hormones, including thyrotropin-releasing hormone (TRH) and the corticotropin-releasing hormone (CRH). The ventrally contiguous anterior periventricular nucleus (aPV) contains somatostatin (SS)-

expressing neurons that also project to the medial eminence. These hormones are transported by the portal vasculature to the anterior lobe of the pituitary where they modulate the secretion of several pituitary hormones. The production of TRH, CRH and SS defines three distinct cell types (fig. 6).

3.) A group of parvocellular neurons project axons to the dorsal motor nucleus of the vagus nerve (DMV), located in the brainstem, which itself projects to virtually all parasympathetic neurons innervating the proximal gastrointestinal (GI) tract, liver and the pancreas through the vagus nerve (Penicaud et al., 2000). These vagal efferents induce gastric secretion and motility, contributing to the feeling of hunger. Finally, the PVN also projects to the nucleus of the solitary tract (NST), located adjacent to the DMV, which receives vagal afferences originating from the GI tract (Saper et al., 1976; Sawchenko & Swanson, 1982; Swanson & Kuypers, 1980; Rogers & Nelson, 1984; Lawrence & Pittman, 1985; Hornby & Piekut, 1988; Hardy, 2001). The NST integrates signals provided by these afferences to modulate the activity of the DMV directly, through some axonal projections (Sawchenko, 1983). The DMV, GI tract and the NST thus form a loop (fig. 7). In vivo electrophysiological studies have shown that the PVN can modulate the activity of gastric distention-responsive NST and DMV neurons (Zhang et al., 1999). The critical importance of the PVN in the control of feeding behaviour is substantiated by the classical observation that its selective destruction leads to hyperphagia (Elmquist et al., 1999).
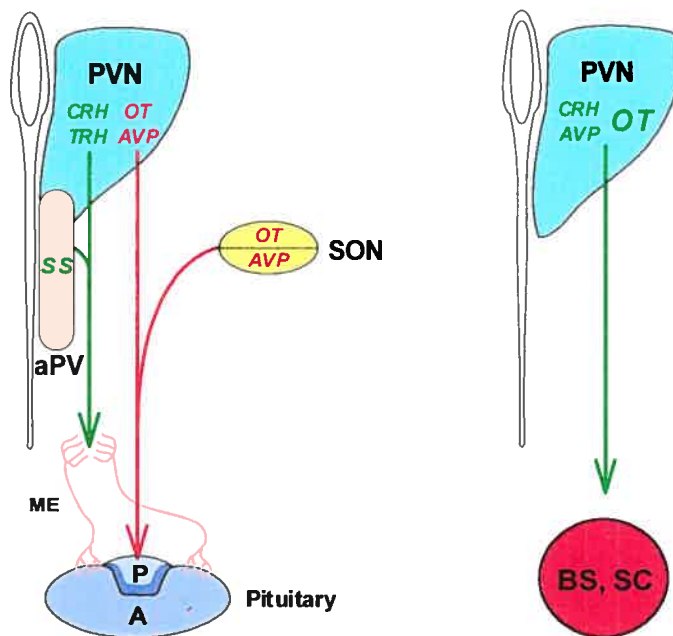
Figure 6. Structural and functional relationship between hypothalamic nuclei. Magnocellular neurons (in red) in the PVN and SON project to the posterior pituitary, whereas parvocellular neurons (in green) of the PVN and aPV producing TRH, CRH and SS project to the medial eminence (ME). In addition, same parvocellular neurons of the PVN can project to preganglion neurons of autonomic nervous system located in the brainstem (BS) and spinal cord (SC). OT expressing neurons represent about 10-20% of these neurons. P: posterior; A: anterior.
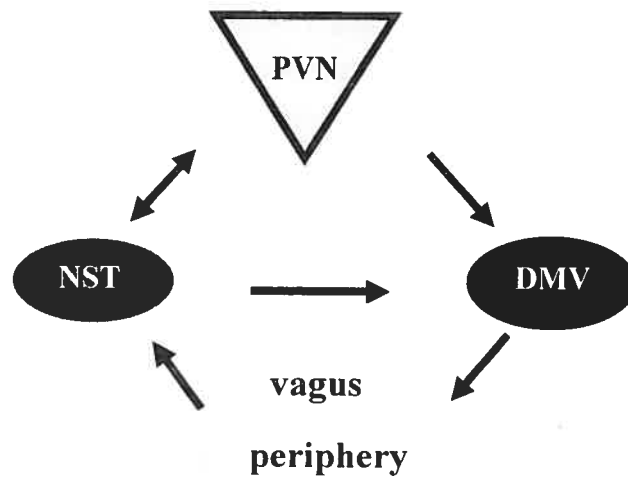
Figure 7. Interaction between the hypothalamus, the brainstem and the periphery. The dorsal medial nucleus of the vagus (DMV) influence gastric motility and secretion through the vagus nerve. Sensitive afferent fibres of the vagus originating from the gut project to the nucleus of the solitary tract (NST). The DMV, the vagus nerve and the NST form a regulatory loop. The PVN appears to regulate this loop through projection to the DMV and NST. Moreover, the PVN can indirectly perceive signals originating from the periphery through projection from the NST.

3.2. Regulation of energy banlance by the paraventricular nucleus

Adiposity signals provide the hypothalamus with information concerning the long-term status of the adipose mass. Leptin is the prototype of this type of signal (Friedman & Halaas, 1998). The current paradigm is that an increase in the adipose mass results in an increased production of leptin which then feeds back in the hypothalamus to decrease food intake and increase energy expenditure. Mutations in leptin or in its receptor cause obesity in human and in mice (Coleman, 1978; Clement et al., 1998; Montague et al., 1997). Leptin has been

shown to act in the arcuate nucleus (ARC) of the hypothalamus (Elias et al., 1999). Leptin decreases the expression of *Npy* and *Agrp* transcripts in medial cells and increases the expression of cocaine- and amphetamine-regulated transcript (*Cart*) and melanocyte-stimulating hormone (*Msh*) transcripts in lateral cells of the ARC (Elmquist, et al., 1999). Genetic and physiological studies indicate that both neuropeptide Y (NPY) and agouti-related protein (AgRP) increase food intake and decrease energy expenditure (Billington et al., 1991; Graham et al., 1997; Ollmann et al., 1997) whereas CART and MSH, which is derived from pro-opiomelanocortin (POMC), have the opposite effects (Fan et al., 1997; Kristensen et al., 1998). These changes of expression are expected to generate a similar physiological response as that of leptin, decrease of food intake and increase of energy expenditure.

ARC neurons expressing *Nyp/Agrp* and *Cart/Msh* project to the PVN (Elmquist et al., 1999; Elias et al., 1999) (fig. 8). Electrophysiological studies have shown that individual neurons within the PVN are capable of detection and integration of the NPY and MSH signals (Cowley et al., 1999). AgRP and MSH are in fact antagonist and agonist, respectively, of *Mc4R*, which is expressed in the PVN (Ollmann et al., 1997; Fan et al., 1997). The importance of the melanocortin pathway is illustrated by the identification of mutations in *Pomc* (Krude et al., 1998) and *Mc4R* (Vaisse et al., 1998; Yeo et al., 1998) in patients with extreme obesity. Moreover, injections of NPY and MSH into the PVN have antagonistic effects on feeding and energy balance (Cowley et al., 1999).

A second class of peripheral signals provides information to the hypothalamus about the short-term nutritional status of the organism. These signals, generally produced by the GI tract in response to feeding, include peptides such as cholecystokinin (CCK) (Fan et al., 2004; Wynne et al., 2005) and nutriments such as linoleic acid (Randich et al., 2004). Several of these signals have been shown to induce satiety by acting directly on the ARC or by modulating the activity of the NST through vagal afferents (Fan et al., 2004). The PVN can sense some of

these signals through projections from the NST. All together, these observations indicate that the PVN has the potential of integrating peripheral short and long-term signals providing information about the nutritional status of the organism and of modulating energy balance through its projections to the NST and preganglionic neurons of the autonomic nervous system.
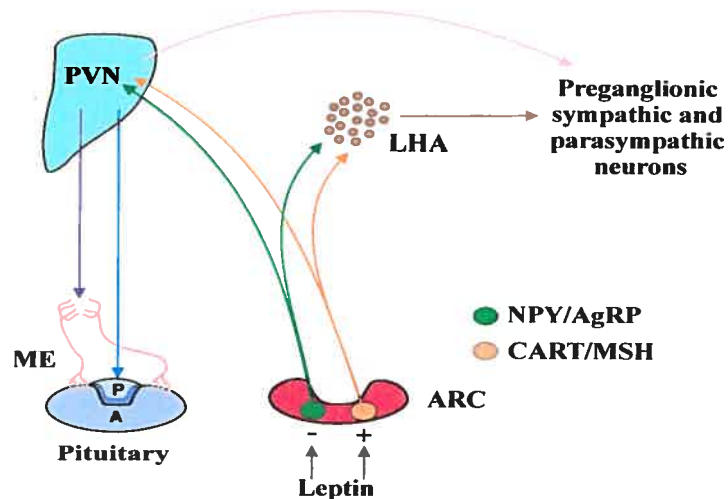
Figure 8. Interaction between the arcuate nucleus (ARC) and the PVN. The ARC is the main hypothalamic site of leptin action. In the ARC, leptin activate CART/MSH cells and inhibit NPY/AgRP cells which project to the PVN and lateral hypothalamic area (LHA). (Caqueret et al., 2005)

Some observations suggest that regulation of food intake and energy expenditure by the PVN involves divergent pathways. For instance, NPY injection into the PVN induces feeding but it also decreases energy expenditure by decreasing the sympathetic outflow to brown adipose tissue. Both the feeding-stimulatory and brown fat-inhibitory effects of NPY in the PVN are blocked by high doses of opioid antagonist administered into the rostral NST (rNST). However, at lower

doses of opioid antagonist, the feeding, but not the brown fat-inhibitory, effects of NPY in the PVN are blocked. These findings suggest that feeding signals originating on stimulation of NPY receptors in the PVN are routed through opioid pathways in the rNST, whereas signals conveying energy expenditure information may be directed through opioid receptors present in neural centers nearby or within other regions of the NST (Kotz et al., 1998). More recenly, a loxP modified, null *Mc4r* allele (loxTB *Mc4r*) was generated. *Mc4r* gene codes a melanocortin receptor. Mice homozygous for the loxTB *Mc4r* allele do not express MC4Rs and are markedly obese. Restoration of MC4R expression in the PVN and a subpopulation of amygdala neurons, using *Sim1*-Cre transgenic mice, prevented 60% of the obesity. The increased food intake of *Mc4r* null mice was completely rescued while energy expenditure was unaffected. These findings demonstrate that the melanocortin pathway in the PVN and/or the amygdala controls food intake but that a melanocortin pathway located elsewhere controls energy expenditure (Balthasar et al., 2005).

4. Function of *Sim1* and *Sim2* in the paraventricular nucleus and in other parts of the brain

4.1. Roles of *Sim1* in the control of neuron differentiation in PVN/SON/aPV

Mutant analyses have demonstrated that *Sim1* functions to control development of specific cell types within the CNS like its *Drosophila* counterpart. PVN, aPV and SON cells expressing the neuropeptides TRH, SS, CRH, VP, and OT, define at least five major neuroendocrine cell types (fig. 9, Michaud et al., 1998). *Sim1* is required for the development of these hypothalamic neurons acting at the final stages of their differentiation. Since *Sim1* is expressed in virtually all PVN cells, it appears likely that its development is abolished in *Sim1*$^{-/-}$ mice. *Sim1* functions upstream to maintain *Brn2* expression. BRN2, a POU transcription factor, in turn directs the terminal differentiation of a subset of cells identified by the production of CRH, OT and VP. SIM1 also acts in parallel with the transcription factor

orthopedia protein (OTP) for the development of the same lineages as those specified by SIM1 (Acampora et al., 1999; Wang & Lufkin, 2000). Yeast two hybrid studies do not provide evidence that SIM1 and OTP interact directly (Yang & Michaud, unpublished results). Several observations indicate that ARNT2 is the in vivo dimerization partner for SIM1: 1.) *Sim1* and *Arnt2* are co-expressed in the developing PVN/SON; 2.) SIM1 and ARNT2 can physically interact in yeast two-hybrid and co-immunoprecipitation assays; 3.) *Sim1* and *Arnt2* mutant mice show the same phenotype (Keith et al., 2001; Hosoya et al., 2001); and 4.) *Sim1* and *Arnt2* function at the same stage of development (Michaud et al., 2000; Ema et al., 1996; Goshu et al., 2004).

4.2. Role of *Sim1* in energy balance

Sim1 homozygous mice (*Sim1$^{-/-}$*) die perinatally, presumably from the PVN developmental defect. In contract, *Sim1* heterozygous mice (*Sim1$^{+/-}$*) survive and develop early-onset obesity with increased linear growth, hyperinsulinemia and hyperleptinemia. These mice are hyperphagic, even before they show increased weight gain, but their energy expenditure is not significantly decreased (Michaud et al., 2001; Holder et al., 2004). A balanced translocation interrupting *SIM1* was found in a child with a similar phenotype (Holder et al., 2000). Like *Sim1$^{+/-}$* mice, this child had early-onset obesity, increased linear growth, and a voracious appetite suggestive of hyperphagia. Mouse models suggest that *SIM1* haploinfficiency is responsible for the obesity in this child. The description of morbid obesity in children with chromosomal deletions in the 6q16 region, which contains *SIM1*, further strengthens this conclusion (Villa et al., 1995; Turleau et al., 1988; Gilhuis et al., 2000). Interestingly, a genome-wide search for childhood obesity traits has shown significant linkage on chromosome 6q22-q23, near the *SIM1* locus (Meyre et al., 2004).

The mechanisms underlying the hyperphagia associated with *Sim1* haploinsufficiency are still under investigation. *Sim1* expression is not detected in

the adult liver, pancreas, muscle, white adipose tissue (WAT) and brown adipose tissue (BAT). Also, *Sim1* is not expressed in the brainstem and in the intermediate/dorsal spinal cord where autonomic preganglionic neurons are found. All these information do not support the possibility that *Sim1* functions in the peripheral tissues to regulate energy balance. One possibility is that dysfunction of the PVN causes this hyperphagia. This dysfunction could be of developmental origin. Indeed, the PVN of *Sim1$^{+/-}$* mice is hypocellular, containing 24 % fewer cells (Michaud et al., 2001). However, since *Sim1* and its dimerizing partner *Arnt2* are strongly expressed in the PVN not only during its development but also throughout adult life, the possibility that *Sim1* controls food intake physiologically is not excluded. Finally, it is also possible that *Sim1* regulates food intake by acting in other regions of the brain such as scattered cells located in the anterior portion of the lateral hypothalamus or in the amygdale.
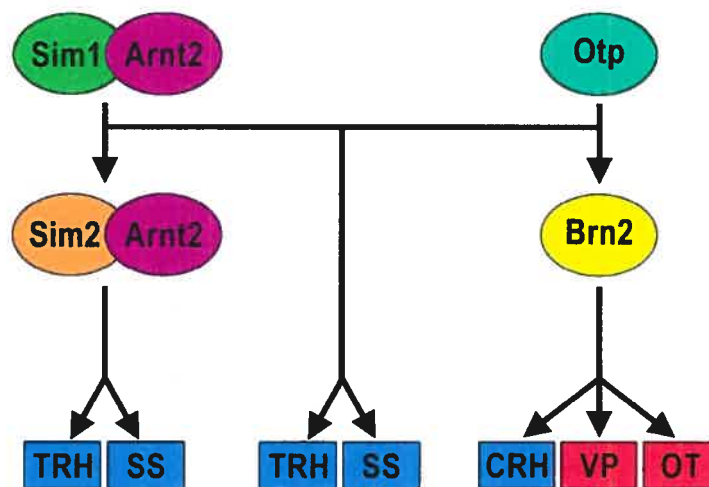
Figure 9. Transcriptional regulation of anterior hypothalamic development. SIM1-ARNT2 and OTP function in parallel to control the development of virtually all neurons of the PVN/SON, of TRH-producing neurons of the preoptic region and of SS-producing neurons of the anterior periventricular nucleus, which is located ventrally contiguous to the PVN. These two complexes are required to

maintain *Brn2* expression, which, in turn, directs the development of CRH-, OT- and VP-producing neurons of the PVN and SON. SIM1-ARNT2 and OTP also act upstream of SIM2 for the development of subsets of TRH cells located in the PVN and preoptic region and of SS cells found in the anterior periventricular nucleus.

4.3. *Sim2*, a paralog of *Sim1*

$Sim2^{-/-}$ mice die within 3 days of birth due to respiratory failure, exhibiting reduced efficacy of lung inflation and several abnormalities involving the thorax (Goshu et al., 2002). *Sim2* regulates the growth or integrity of the ribs and vertebrae, although its precise function remains unclear. *Sim2* is also expressed in the anterior hypothalamus (Wang & Lufkin, 2000; Goshu et al., 2004). Along the rostro-caudal axis, *Sim2* expression is detected in the anterior and mid-PVN, including the aPV. Toward the posterior PVN, *Sim2* expression diminishes. In these anterior areas, *Sim2* expression domain correlates with that of *TRH* and *SS* expressing cells and is distinct from that of *Brn2*, which is necessary for the differentiation of *OT, VP* and *CRH* neurons located more posteriorly. In the absence of *Sim1* function, there is no *Sim2, TRH,* and *SS* expression, reflecting a general and upstream role of *Sim1* (Goshu et al., 2004). In the absence of *Sim2* function, *TRH* and *SS* cells are reduced even with normal *Sim1* gene dosage, reflecting a semidominant and downstream role of *Sim2*. Furthermore, in the absence of *Sim2* function, *Sim1* dosage has an influence on *TRH* and *SS* cell numbers, demonstrating that *Sim1* has some capacity to compensate for *Sim2* (Goshu et al., 2004). These results demonstrated that *Sim2* acts downstream of *Sim1* and contributes to neuroendocrine hormone gene expression in the anterior hypothalamus. Unlike *Sim1, Sim2* does not regulate energy balance, because $Sim1^{+/+};Sim2^{+/-}$ mice do not show obesity and $Sim1^{+/-};Sim2^{+/-}$ mice are not more obese than $Sim1^{+/-}$ mice. Thus the loss of one allele of *Sim2* does not cause

obesity on its own, nor does it enhance the $Sim1^{+/-}$ phenotype (Goshu et al., 2002).

Human *SIM2*, was first identified by exon trapping of a region of the human chromosome 21 (Chen et al., 1995; Dahmane et al., 1995) that is known to be associated with many of the pathological features of Down syndrome (DS) (Delabar et al., 1993). Its homolog was subsequently found to be located at the syntenic region on chromosome 16 in the mouse (Moffett et al., 1996). The expression pattern of mouse *Sim2* in the brain, craniofacial structures, and muscles suggests that it may contribute to the mental retardation, facial dysmorphology, and hypotonia of DS patients (Dahmane et al., 1995; Fan et al., 1996; Yamaki et al., 1996). This is supported by the observation that transgenic mice trisomic for *SIM*2, with one BAC-containing copy of *SIM2*, and two endogenous copies of *Sim2*, show some aspects of Down's phenotype, exhibiting anxiety-related/reduced exploratory behaviour and reduced sensitivity to pain (Chrast et al., 2000).

4.4. *Sim1 and Sim2* function during mammillary body development

4.4.1. Structure and function of the mammillary body

MB is composed of a small number of nuclei located at the caudal end of the hypothalamus. Most MB neurons send axonal projections to both the anterior thalamic nuclei (mammillothalamic tract, MTT) and the tegmentum (mammillotegmental tract, MTEG) (fig. 10, Marion et al., 2005). In the mouse, the development of this system of fibres was studied by Tello (1936–1937). Mammillary projections traveling through the principal mammillary tract (PMT) are established early during development, forming the mammillotegmental bundle, which appears fully developed by embryonic day 15 (E15). The mammillothalamic tract develops later, around E17–E18, forming a compact system of collateral fibres originating from the principal mammillary tract and reaching the thalamus during the first postnatal days (Valverde et al., 2000).

Thus, mammillothalamic bundle represents a branch of bifurcation of the principal mammillary tract.

The MB and its axonal projections MTT and MTEG are components of the classic circuit of Papez, which is involved in spatial learning and memory processing (Milner, 1970; Weiskrantz, 1978; Squire & Zola-Morgan, 1991). For instance, selective destruction of the MB in rat induces impairment of spatial learning and memory (Saravis et al., 1990; Sziklas & Petrides, 2000). Moreover, severe anterograde amnesia in patients with Korsakoff's psychosis is associated with injury of the mammillary bodies (Kahn & Crosby, 1972; Mayes et al., 1988; Kopelman, 1995).
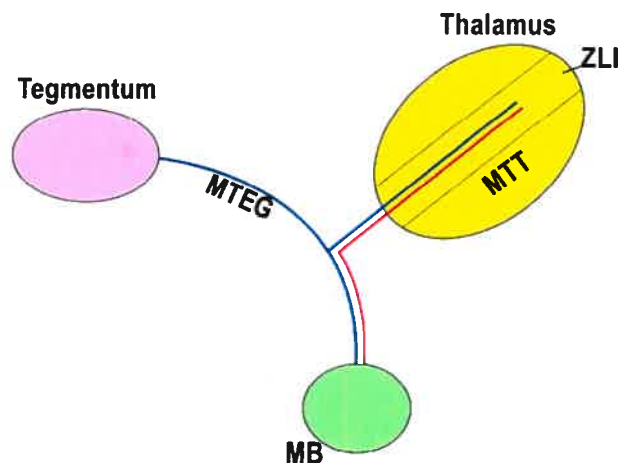
Figure 10. Axonal projection originating from the mammillary body.   Rostral is to the right. The principal mammillary tract (PMT) gives rise to the mammillotegmental (MTEG) and mammillothalamic tract (MTT) (Marion et al., 2005). In the developing thalamus, the MTT axons appear to be induced at the land of the zona limitans intrathalamica (ZLI).

4.4.2. Transcription factors controlling mammillary body development

Although nothing is known about the molecular mechanisms involved in guiding the MTEG axons, there is evidence that the thalamus produces signals that attract MTT axons. The MTT, but not the MTEG, fails to form in mice deficient in the transcription factor PAX6 (Valverde et al., 2000). During development, *Pax6* is not expressed in the MB but it is expressed in the ventral thalamus. The winged helix FOXB1 transcription factor is also involved in MTT formation (Alvarez-Bolado et al., 2000; Kloetzli et al., 2001). *Foxb1* mutant mice are born with an intact MTEG but without a MTT. *Foxb1* is expressed in the MB and in a dorsal thalamus domain. Failure of MTT formation in *Foxb1* mutants is associated with apoptosis of the MB perinatally, possibly induced by the absence of connections to the thalamic targets (Alvarez-Bolado et al., 2000). The requirements for MTT axon guidance are thus complex, since the signals controlled by *Pax6* and *Foxb1* are produced by closely located but non-overlapping regions of the thalamus.

MB neurons are born between E10.5 and E13.5 in mice (Altman & Bayer, 1978). Previous studies have shown that *Sim1* and *Sim2* start to be expressed in the neuroepithelium that will give rise to the MB at E9.5, before the birth of its first neurons (Fan et al., 1996). In our recent study of *Sim1* and *Sim2* in the developing MB, we have found that: a.) MB neurons are generated and survived at least until E18.5 in embryos lacking both *Sim1* and *Sim2* ($Sim1^{-/-};Sim2^{-/-}$); b.) MTT and MTEG are histologically absent in $Sim1^{-/-};Sim2^{-/-}$ embryos and reduced in embryos lacking *Sim1*, but bearing one or two copies of *Sim2*; in place, abnormal MB axons grow towards the midline. Interestingly, the expression of *Rig-1/Robo3*, a negative regulator of *Slit* signalling, is up-regulated in the prospective MB of *Sim1/Sim2* double mutants. SLIT1 and SLIT2 are secreted molecules that induce repulsion of axons that express its receptor ROBO1 or ROBO2 (Bagri et al., 2002). *Slit1 and Slit2* are produced in the ventricular layer adjacent to and dorsal to the MB. *Rig-1/Robo3* is a distant homolog of *Robo1* and *Robo2* that appears to function cell autonomously to inhibit SLIT signalling (Sabatier et al.,

2004; Marillat et al., 2004). *Rig-1* is expressed ectopically in the developing MB of *Sim1/Sim2* double mutants raising the possibility that it contributes to the axonal defects by decreasing the sensitivity of MB axons to *Slit* signalling (Bagri et al., 2002). It is noteworthy that *Sim1* and *Sim2* act along compensatory, but not hierarchical pathways in the developing MB (Marion et al., 2005). Taken together, our results suggest that *Sim1* and *Sim2* are required for MB axonal development and play similar roles in vivo.

5. Hypothesis and objectives

The bHLH-PAS transcription factor *Sim1* is required for the final stages of differentiation of hypothalamic neurons located in the paraventricular nucleus (PVN). The PVN is a region of the hypothalamus that regulates appetite and energy expenditure. Haploinsufficency of *Sim1* causes hyperphagia and obesity in mice and humans. Several observations suggest that this hyperphagia results from some dysfunction of the PVN. We found that the PVN of *Sim1*$^{+/-}$ mice is hypocellular, suggesting that PVN dysfunction could be secondary to a developmental mechanism. However, since *Sim1* and its function partner *Arnt2* are also expressed in PVN neurons in adulthood, it also could function in the postnatal PVN to control food intake physiologically. In order to explore this hypothesis, we propose to: 1) use transgenesis to specifically modulate *Sim1* expression in the PVN. Because a PVN specific element is not available at this time, we propose to characterize *Sim1* regulatory elements in order to identify such an element; 2) use an adenoviral vector to produce *Sim1* or a small hairpin RNA directed against *Sim1* in the PVN of adult mice.

CHAPTER II: REGULATORY INTERACTION BETWEEN ARYLHYDROCARBON RECEPTOR AND SIM1, TWO BASIC HELIX-LOOP-HELIX PAS PROTEINS INVOLVED IN THE CONTROL OF FOOD INTAKE

REGULATORY INTERACTION BETWEEN ARYLHYDROCARBON RECEPTOR AND SIM1, TWO BASIC HELIX-LOOP-HELIX PAS PROTEINS INVOLVED IN THE CONTROL OF FOOD INTAKE

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Abbreviations used in the text: PVN, paraventricular nucleus; bHLH, basic helix-loop-helix; PAS, Per, ARNT, AHR, SIM homology domain; AHR, arylhydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DRE, dioxin-responsive element; ARNT, AHR nuclear translocator; HIF, hypoxia-inducible factor; HIF-1α, hypoxia-inducible factor-1α; RACE, rapid amplification of 5'-cDNA ends; TSS, transcription start site;

Abstract

The basic helix-loop-helix PAS (bHLH-PAS) transcription factors SIM1 and arylhydrocarbon receptor (AHR) are involved in the control of feeding behavior. *Sim1* haploinsufficiency causes hyperphagia in mice and humans, most likely by perturbing the hypothalamus function. The administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand of AHR, causes severe anorexia, which also appears to be of central origin. Both SIM1 and AHR require heterodimerization either with ARNT or ARNT2 to function. Here, we characterize the promoter for *Sim1* and show that a consensus AHR-ARNT/2 binding site positively regulates its activity in the context of transfection experiments in Neuro-2A cells. A gel shift assay indicated that AHR-ARNT/2 can bind its putative site in the *Sim1* promoter. Overexpression of *Arnt*, *Arnt2*, or *Ahr* increased the activity of a reporter construct containing the *Sim1* promoter by 1.8-, 1.5-, and 2.2-fold, respectively, but failed to do so when the AHR-ARNT/2 binding site was mutated. Similarly, TCDD increased the activity of the reporter construct by 1.9-fold but not that of its mutated version. Finally, we found that TCDD increased *Sim1* expression in Neuro-2A cells and in mouse kidney and hypothalamus by 4-, 3-, and 2-fold, respectively. We conclude that *Sim1* expression is regulated by AHR-ARNT/2. This result raises the possibility that *Sim1* mediates the effect of TCDD on feeding and points to a complex network of regulatory interactions between bHLH-PAS proteins.

Introduction

The paraventricular nucleus (PVN) of the hypothalamus contains neuroendocrine cells that are critical for the regulation of several physiological processes, including energy balance and blood pressure. Loss-of-function experiments in mice have shown that the bHLH-PAS transcription factor SIM1 is essential for the differentiation of PVN neurons (1). In the absence of *Sim1*, virtually all PVN neurons fail to develop. Interestingly, whereas *Sim1*-homozygous mice die shortly after birth, presumably from the PVN defect, *Sim1* heterozygous mice survive and develop hyperphagia and obesity (2). Also, a balanced translocation interrupting *SIM1* was found in a child with isolated hyperphagia and severe obesity (3). Since the PVN is a key regulator of appetite and *Sim1* is not expressed in other nuclei involved in the control of feeding, it has been proposed that PVN dysfunction causes the hyperphagia associated with *Sim1* haploinsufficiency. Such a PVN dysfunction could be related to the developmental function of *Sim1*. Indeed, the PVN of *Sim1* heterozygous mice is hypocellular (2). On the other hand, *Sim1* is continuously and strongly expressed in the mature PVN, raising the possibility that it could also control feeding behavior physiologically.

The arylhydrocarbon receptor (AHR), another bHLH-PAS protein, also has the potential of affecting feeding behavior. The subcellular localization of AHR is determined by its interaction with small ligands, which include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a toxic aromatic hydrocarbon produced in our industrial environment. Upon binding to TCDD, AHR is translocated from the cytoplasm to the nucleus where it activates transcription of target genes (reviewed in Refs. 4–6). Administration of a single sublethal dose of TCDD to rodents specifically induces anorexia without causing nausea or triggering other forms of behavior (reviewed in Refs. 7 and 8). Other toxic effects of TCDD include thymic involution, immunosuppression, and various endocrine abnormalities. However, decreased food intake is the cause of the weight loss associated with acute TCDD toxicity and is a major contributor to the lethality observed at higher dose. Mutant

analysis has confirmed that TCDD toxicity requires *Ahr* (9). The mechanism by which TCDD induces anorexia remains unknown, but there are some evidences that it might involve the hypothalamus (reviewed in Refs. 7 and 8).

SIM1 and AHR belong to a group of proteins that need to heterodimerize with members of another group of bHLH-PAS proteins, of which only four representatives are as yet characterized: ARNT (10), ARNT2 (11), BMAL1/MOP3 (12–15), and BMAL2/MOP9 (16–19). SIM1 and AHR each can physically interact with ARNT, ARNT2, or BMAL1/MOP3 to form heterodimers (4, 11, 20). The ability of SIM1 or AHR to interact with BMAL2/MOP9 has not been reported. Mutant studies have established that heterodimerization of SIM1 with ARNT2 is required for PVN development (20–22). Although SIM1 and AHR interact with the same partners, they appear to bind different DNA sequences (23, 24). Systematic investigations of DNA binding specificities of bHLH-PAS proteins have shown that a heterodimer composed of *Drosophila* SIM or mammalian SIM1 and ARNT preferentially binds to GT(G/A)CGTG, whereas mammalian AHR-ARNT preferentially binds to TNGCGTG, which defines the so-called dioxin-responsive element (DRE) (24–27).

Here, we characterize the *Sim1* promoter and show that its activity is positively regulated by AHR-ARNT/2 and TCDD in cultured cells as well as in mice. These observations raise the possibility that *Sim1* mediates the effect of TCDD on feeding behavior and suggest the existence of complex regulatory interactions between bHLH-PAS proteins.

Experimental Procedures

Constructs

Sim1 genomic fragments were generated by PCR from a mouse BAC clone and were inserted into pGL3-basic and pGL3-promoter expression vectors which contain a luciferase reporter gene (Promega Corp, Madison, WI). The DRE located in *Sim1* 5' region was mutated by using the QuikChange Site-Directed

Mutagenesis Kit (Stratagene). *Arnt* and *Ahr* expression vectors were generous gifts from Drs. Yoshiaki Fujii-Kuriyama and Christopher Bradfield, respectively. The *Arnt2* expression vector has been previously described (20).

Chemical

TCDD was purchased from The National Cancer Institute Chemical Carcinogen Repository Midwest Research Institute. For cell culture experiments, it was dissolved in DMSO and added to culture media at a final concentration of 10nM when 40% confluence was reached (28-31). Cells were harvested after 48 hours of incubation. For animal experiments, TCDD was dissolved in a corn oil/acetone mix (95%/5%, v/v) and administered intraperitoneously at a dose of 360 μg/kg to 5 month-old C57BL/6 male mice.

RACE

Rapid amplification of 5'cDNA ends (RACE) analysis was performed by using the CLONTECH SMART RACE cDNA amplification kit according to the manufacturer's protocol. Briefly, first-strand cDNA synthesis was performed using 1ug of total RNA. Touchdown PCR was then performed with *Sim1* gene specific primer GSP1 (5' GTCCAGTTGGGAGGTGATGGCTGAGGG-3'). Nested PCR was performed with primers GSP2 (5'-CCGGGAAGACACG AATGATGGACAGG-3') or GSP3 (5'-CCGCGGTAAGAACCCGGGTCCTTG GGA-3'). The nested PCR products were cloned into pLivSelect-PCR Cloning vector (Bio S&T Inc, Montreal, QC), and sequenced.

Cell transfection and luciferase assay

The Neuro-2A cell line was purchased from American Type Culture Collection (Rockville. MD). Neuro-2A (N2A) cells were maintained in minimum essential medium Eagle with 10% fetal bovine serum, 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/Liter sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0mM sodium pyruvate. Cells were transfected with Lipofectin (Invitrogen) according to instructions from the manufacturer. Transfection was performed 24 hours after seeding 1×10^5 cells/well in 6-well

tissue culture dishes using 2.0 µg of plasmid. In co-transfection experiments, 1.5 µg of each plasmid was used. All transfection experiments also included the pHβ plasmid (1 µg), which produces β-galactosidase. After overnight incubation, media was changed and cells were cultured for 48 hours. Cells were then scraped from the dishes, pelleted by centrifugation and resuspended in lysis buffer. Luciferase activity was measured using the Luciferase Assay System (Promega) according to the manufacturer. Aliquots of the lysate were used for assay of β–galactosidase activity to control for transfection efficiency. Luciferase activity was expressed as relative activity compared with that of control vectors and shown as the mean +/- SD of three independent transient transfection experiments, each performed in duplicate.

Electrophoretic Mobility Shift Assay (EMSA)

Mouse AHR, ARNT2, and SIM1 proteins were synthesized *in vitro* using the TNT-coupled reticulocyte lysate system (Promega). The reactions were carried out for 90 min at 30 °C. The relative amounts of the translated proteins were semi-quantified by visualization on 10% SDS-PAGE after incorporation of biotin-lysyl-tRNA (Promega).

Electrophoretic mobility shift assay was done as described by Swanson *et al.* (27). Briefly, equal amounts of dimerizing partners were incubated for 2 h at 30 °C in the presence or absence of TCDD (0.4 µM). Poly(dI-dC) (200 ng or 1.2 mg) and KCl (final concentration: 100 mM) were added to the protein mixture in a final volume of 10 µl, and incubation was carried out at room temperature for 10 min. In some instances, a 500-fold excess of unlabeled oligonucleotide was also added. End-labeled double-stranded oligonucleotide (100,000 cpm) was then added, and the sample was incubated for an additional 10 min at room temperature. The reaction mixture was submitted to electrophoresis on a 4% acrylamide nondenaturing gel using 0.5x TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0) as the running buffer. The gel was dried and autoradiographed. Each electrophoretic mobility shift assay experiment was performed

independently three times. The nucleotide sequences of the oligonucleotides used were: D1, 5'-TCGAGCTGGGGGCA<u>TTGCGTGA</u>CATACC-3'; D2, 5'-TCGAGG TATGT<u>CACGCAA</u>TGCCCCCAGC-3'; S1, 5'-AGCCGCGAGCCCAG <u>TCGCGTG</u>GGGGG-3'; S2, 5'-CCCCCCCCC<u>ACGCGA</u>CTGGGCTCGCG-3'; M1, 5'-AGCCGCGAGCCCAG<u>TC</u>GGGGGG-3'; M2, 5'-CCCCCCCC<u>GA</u>CT GGGCTCGCG-3'. Oligonucleotides D1 and D2 contain a prototypical DRE that was characterized by Swanson *et al.* (27).

<u>Reverse Transcriptase-PCR Analysis</u>

Total RNA was isolated using TriPure Isolation Reagent (Roche Diagnostics, Laval, Quebec, Canada). Single-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Reverse transcriptase-PCR for detection of *Ahr* (32), *Arnt* (33), and *Arnt2* (34) expression was performed as described previously.

Primers for real-time PCR were designed using Primer3 software (Whitehead Institute for Biomedical Research), subjected to a melting curve analysis, and then examined by standard PCR and agarose gel electrophoresis for correct product size. Quantitative PCR was performed using a Smart Cycler (Cepheid). For each reaction, 25 μl of SYBR-Green PCR, 1 μl of cDNA, 1 μl of each primer (5 μM), and 12.5 μl of SYBR-Green JumpStart Taq Ready Mix (Sigma) were mixed together. *Sim1* PCR was carried out at 94 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s, for 45 cycles. The same conditions were used to amplify β-actin with the exception that the annealing temperature was 55 °C. Real-time PCR reactions using the same starting amount of RNA purified from the different tissues studied showed that TCDD did not change the level of β-actin expression. For each sample, reactions were performed in duplicate, and threshold cycle numbers were averaged. *Sim1* expression was normalized to β-actin and fold induction was calculated according to the formula described by Buckhaults *et al.* (35). The sequences of the primers used are: *Sim1*, 5'-CAAAATGAGAGT GGTCTTTCCAG-3' and 5'-GGAGTAGATGAGAGCCGAGTTC-3'; β-actin, 5'-

ACGTTGACATCCGTAAAGACCT-3' and 5'-GCAGTAATCTCCTTCTGCA TCC-3'.

Results

A 5'RACE approach was used to map *Sim1* transcription start site (TSS). A first round of amplification using primer GSP1, which sequence is located in the translated region, was followed by a round of nested amplification with either primer GSP2 or GSP3, located in the 5'-untranslated region (Fig. 1A). Nested PCRs using primers GSP2 and GSP3 generated fragments of 560bp and 340bp, respectively, from murine newborn kidney (Fig. 1B). Fragments of similar size were also obtained at 12.5 days post-coitus and newborn hypothalamus (data not shown). Sequencing of these nested amplification products indicated that the TSS is located 1121 nucleotides upstream of the translation start codon. Sequence analysis showed the presence of consensus TATA, GC and CAAT boxes, respectively, 43, 94 and 133/164 bp upstream of the TSS (Figure 1C). A high level of identity between mouse and human sequences (77%) was found over a segment of 184 bp located immediately 5' to the TSS.

We next cloned a 670 bp fragment (named fragment A), encompassing *Sim1* TSS, upstream of a luciferase reporter gene in the promoterless pGL3-basic vector (Fig. 2A). This construct was transiently transfected into N2A cells, which express *Sim*1, and luciferase activity was assayed (Fig. 2A). Fragment A increased luciferase activity over baseline by 6-fold indicating promoter activity. Deletion of 250 bp at the 3' end of fragment A resulted in a 5-fold increase of luciferase activity, suggesting the existence of an element between position -15 and +235 that inhibits promoter activity (fragment A2; Fig. 2A). Further deleting a 38 bp segment, which contains a potential TATA box, decreased the activity of the promoter by 60% (fragment A3) whereas a larger 3' deletion removing the GC and CAAT boxes abolished promoter activity (fragment A4). Deletion of 322 bp at the 5' end of fragment A was associated with low but detectable promoter

activity (fragment A5). This series of deletion locates *Sim1* minimal core promoter between position -178 and the TSS. Similar results were obtained with the 293 cell line which also expresses *Sim1* (data not shown).

Fragment A4 had no promoter activity. However, fragment A4 contained a positive regulatory element since it increased the activity of a SV40 promoter by more than 3 fold in N2A cells, as shown by luciferase assay (Fig. 2B). Deletion of 47 bp at the 3' end of the A4 fragment resulted in a 50% decrease of this stimulatory activity (Fig. 2b, fragment A6). We noticed that the consensus sequence of the DRE (TCGCGTG) is present in this 47 bp segment. Interestingly, deletion of 4 base pairs within this element (CGTG) decreased the stimulatory activity of fragment A4 on the SV40 promoter by 50%, strongly suggesting that this potential DRE corresponds to the positive regulatory element mapped to the 3' end of fragment A4 (Fig. 2B). Consistently, introduction of the same 4 bp mutation in the A2 fragment (fragment A2m) decreased its promoter activity by 50% (Fig. 2C). Therefore, we identified a potential DRE that positively affects the activity of both SV40 and *Sim1* basal promoters.

It has been shown that the sequence flanking a consensus DRE can affect the binding of the AHR-ARNT(2) complex (25-27). To determine whether the AHR-ARNT complex binds the potential DRE found in fragment A, we performed mobility shift studies using *in vitro* translated AHR and ARNT2. We found that AHR-ARNT2 binds a 26 bp oligonucleotide that contains *Sim1* DRE but not its mutated version (Fig. 3). Binding to *Sim1* DRE was increased in the presence of TCDD, decreased in the presence of an excess of unlabeled oligonucleotide, and unchanged in the presence of unlabeled mutant oligonucleotide (Fig.3; not shown).

To further address the contribution of the DRE to the regulation of *Sim1* expression, the effect of *Ahr, Arnt*, or *Arnt2* overexpression on the *Sim1* promoter activity of fragment A2 was assessed in co-transfection experiments using N2A cells, in which these three genes are expressed (data not shown). Overexpression

of *Ahr, Arnt*, and *Arnt2* increased the promoter activity associated with fragment A2 by 1.8-, 1.5-, and 2.2-fold, respectively, but not that of its mutated version (A2m) in which the DRE has been destroyed (Fig. 4*A*). The addition of TCDD increased by itself the promoter activity associated with fragment A2 by 1.9-fold but had no effect on fragment A2m (Fig. 4*B*). Transfection of the *Ahr* expression vector in the presence of TCDD increased the promoter activity of fragment A2 by 2.4 ± 0.1-fold (not shown); this increase was 28% higher than that obtained in cotransfection experiments performed in the absence of TCDD. Although small, this difference is significant ($p < 0.05$). The lack of an important additive effect of *Ahr* overexpression and TCDD treatment would suggest that a component of the AHR-ARNT/2 complex or of the *Sim1* promoter limits the effect of the DRE element on its activity, at least in the context of the construct used here. Altogether, these results provide functional evidence that AHR-ARNT/2 regulates *Sim1* promoter.

To explore the regulatory interaction between *Ahr* and *Sim1 in vivo*, we studied the effect of TCDD on *Sim1* gene expression in N2A cells using quantitative real-time PCR. We observed that the addition of TCDD to N2A cells increased *Sim1* mRNA levels by 4-fold compared with untreated cells (Fig. 5*C*). We next examined *Sim1* expression in the kidney and hypothalamus of C57BL/6 mice treated with a single dose of TCDD (360 µg/kg), which has been shown to cause severe hypophagia in mice from this strain (36). We confirmed the efficiency of this dose by observing its effects in C57BL/6 mice over a 2-week period (Fig. 5, *A* and *B*). We next measured *Sim1* expression levels in mice tissues 3 days after injection of TCDD or vehicle. A 3- and 2-fold increase of *Sim1* expression in kidney and hypothalamus, respectively, was observed after TCDD administration (Fig. 5*C*). On the whole, we conclude that *Sim1* expression is regulated *in vivo* by AHR-ARNT/2.

Discussion

AHR mediates at least two distinct sets of responses. First, AHR regulates an adaptative response pathway to environmental contaminants such as the polychlorinated dioxins that contaminate industrial chemicals (4-6). Specifically, AHR upregulates xenobiotic metabolizing enzymes, such as CYP1A1, that participate in the degradation and elimination of these toxins. Second, AHR mediates the toxic effects of these compounds. For instance, the administration of TCDD triggers a complex syndrome characterized by thymic involution, immunosuppression, endocrine abnormalities and anorexia. Although it has been shown that *Ahr* is required for the toxicity of TCDD, little is known about the downstream molecular events that lead to its effect (9). Our observation that *Ahr* acts upstream of *Sim1* raises the possibility that the latter is involved in mediating the toxicity of TCDD and points to a complex hierarchal interactions between bHLH-PAS proteins.

TCDD and the control of appetite

The mechanism underlying the effect of TCDD on feeding remains unknown but might involve the hypothalamus keeping with its central role in the control of appetite (reviewed in Ref.7). The observation that the intracerebroventricular injection of TCDD triggers an even stronger effect on appetite than when peripherally administered supports this possibility (37). Several lines of evidence in fact suggest that *Ahr* can act in the PVN, a critical hypothalamic center regulating feeding behavior. First, *Ahr*, *Arnt* and *Arnt2* are expressed in the mature PVN (2;38). Second, TCDD administered peripherally can induce the expression of *Cyp1a1*, a direct target of *Ahr*, in the hypothalamus by 6 fold within 1 day suggesting that it crosses the blood-brain barrier (39). Also, TCDD increases the expression of *c-Fos* in the PVN within 3 days after the administration of a single dose (40). Third, various endocrine perturbations have been observed following the administration of TCDD, some of which were related to processes regulated by the PVN. For instance, chronic administration of

TCDD results in an increase of corticotropin-releasing hormone and vasopressin production by the PVN (41). Also, it has been shown that TCDD can potentiate the secretion of oxytocin by PVN cells (42). Our finding that TCDD increases *Sim1* transcript levels in the hypothalamus further supports the idea that TCDD can modulate gene expression in this structure.

PVN dysfunction appears to underlie the hyperphagia associated with *Sim1* haploinsufficiency. The fact that the PVN of *Sim1* $^{+/-}$ mice is hypocellular suggests that this dysfunction is of developmental origin. On the other hand, *Sim1* is continuously expressed in the PVN postnatally, raising the possibility that it functions physiologically to control feeding. If this is indeed the case, appetite would then be remarkably sensitive to *Sim1* levels because reduction of its gene dosage by 50% is sufficient to markedly increase food intake. Conversely, the 2 fold increase of its expression level in the hypothalamus following TCDD administration would be predicted to decrease feeding. Thus, it is tempting to speculate that the induction of *Sim1* expression by TCDD mediates, at least partially, its effect on feeding. Alternatively, *Sim1* could mediate the effect of TCDD on other aspects of PVN function, such as the secretion of hormones. Also, both the loss of AHR function and the administration of a hydrocarbon ligand of AHR disrupt nephrogenesis (43). *Sim1* is strongly expressed in the developing nephrons and its expression in the kidney increases upon TCDD administration, as shown by our study. It is thus possible that *Sim1* plays a role downstream of *Ahr* in the kidney.

It is unclear whether AHR normally plays a physiological role in the control of feeding behavior in the absence of TCDD. AHR mutant mice were not reported to be thin nor obese although on some background their growth appears transiently decreased (44-46). It will be particularly interesting to determine whether recently identified endogenous ligands for AHR have the same effect on feeding as TCDD and whether they also increases *Sim1* expression (47-51). The analysis of such

natural ligands might reveal some physiological functions of the *Ahr* pathway that were unraveled by mutant analysis.

<u>Regulatory interactions between bHLH-PAS proteins</u>

bHLH-PAS proteins can cross-talk in a parallel manner to regulate target gene expression. First, the activity of two or more bHLH-PAS proteins can be modulated by the availability of a dimerizing partner for which they compete (23;24;52). Second, some bHLH-PAS heterodimers, like HIF-ARNT and SIM1-ARNT, have the potential of binding to the same DNA sequence (24). Third, two bHLH-PAS heterodimers can directly regulate the transcription of the same downstream gene by interacting with distinct regulatory elements, as it was found for AHR-ARNT and HIF-ARNT which both control the expression of the *EPO* gene (53).

bHLH-PAS proteins can also regulate their own expression or the expression of genes coding other bHLH-PAS proteins. For instance, in the fly, SIM positively and directly regulates its own expression through interaction with TANGO, a homolog of ARNT/2. Expression and mutant studies have shown that ARNT2 acts as the dimerizing partner of SIM1 during PVN development (20-22). Our results indicate that ARNT2 can also interact with *Sim1* in a hierarchal manner by forming a complex with AHR that binds a consensus DRE upstream of the *Sim1* promoter. However, we propose that AHR-ARNT2 is not required for basal or tissue-specific expression of *Sim1* but provides an additional layer of regulation that modulates *Sim1* levels. This mechanism of regulation could be critical for the physiological processes controlled by *Sim1* or for TCDD toxicity.

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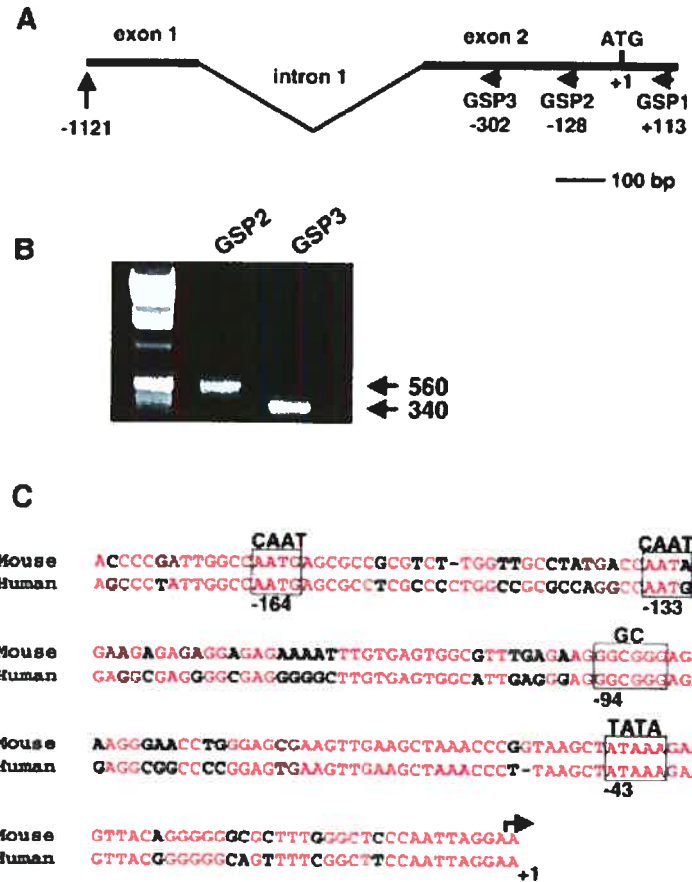
Figure 1.

Figure 1. Identification of *Sim1* transcription start site by 5'-RACE analysis.
A, scheme showing the position of the primers used for the 5'-RACE analysis. A RACE fragment generated with primer GSP1 served as a template in nested PCR reactions using either primer GSP2 or GSP3. *B,* agarose gel showing nested PCR products of 560- and 340-bp amplified with primer GSP2 or GSP3, respectively, from newborn kidney tissue. The size of these fragments and their sequences indicate that the *Sim1* transcription start site is located 1121 bp upstream of the translation initiation codon. *C,* comparison of mouse and human genomic sequences located immediately 5' of the transcription start site. The identity between the two sequences is 77% over a stretch of 184 bp. Potential TATA, GC, and CAAT boxes are outlined.
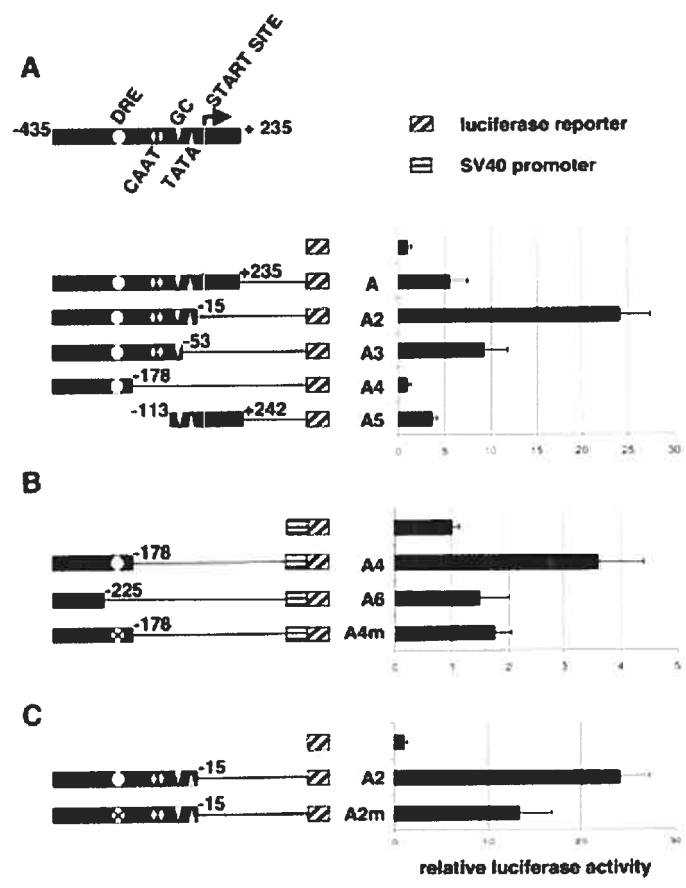
Figure 2.

Figure 2. Identification of a functional DRE in *Sim1* promoter region.

A, promoter activity of *Sim1* genomic fragments. A 670-bp fragment encompassing the *Sim1* transcription start site or various subfragments were cloned upstream of a luciferase reporter gene in the pGL3-basic vector. These different constructs were transiently transfected in N2A cells and the luciferase activity was measured. Relative luciferase activities are expressed as-fold induction over the value obtained from transfection of the pGL3-basic empty vector. Results were significantly different when compared between each other (*p* < 0.01) with the exception of the comparison between vector A4 and the control vector (*p* > 0.5). *B,* effect of *Sim1* genomic fragments on the activity of the SV40 promoter. *Sim1* genomic fragments were cloned upstream of the SV40 promoter and of a luciferase reporter gene in the pGL3-promoter vector. These constructs were transiently transfected in N2A cells. Relative luciferase activities are expressed as-fold induction over the value obtained from transfection of the pGL3-promoter vector which contains the SV40 promoter and the luciferase reporter gene but no *Sim1* sequence. Comparison between results obtained with vector A4 and the other vectors showed significant difference (*p* < 0.01), whereas comparison between results obtained with vectors A4m, A6 and the control vectors showed no significant difference (*p* > 0.05). *C,* loss of a potential DRE reduced *Sim1* promoter activity. Results were significantly different when compared between each other (*p* < 0.001). Results represent means ± S.D. of three independent experiments, each performed in duplicates. Statistical analysis was performed with a Student *t* test.
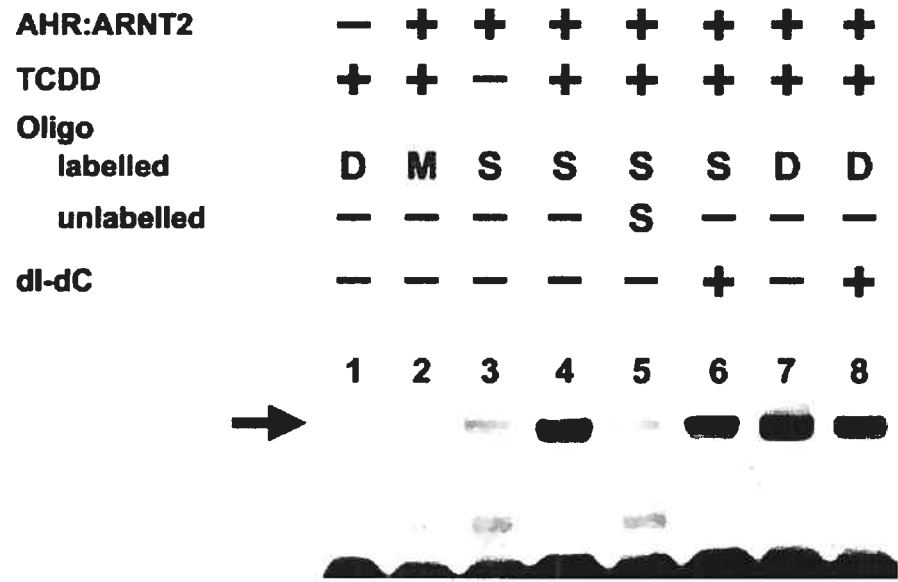
Figure 3.

Figure 3. Binding activity of AHR-ARNT2 to the *Sim1* DRE.

AHR and ARNT2 were produced by *in vitro* transcription and translation, and comparable amounts of each protein were incubated with labeled oligonucleotides containing *Sim1* DRE (*S*), mutated *Sim1* DRE (*M*), or a prototypical DRE (*D*) in the presence or absence of TCDD (0.4 μM), 500-fold excess of unlabeled *Sim1* DRE oligonucleotide, or a high concentration of dI-dC. The band corresponding to the oligonucleotide-heterodimer complex is indicated by an *arrow*. The experiment was performed three times independently.

A

A2 ■ A2m ▨

relative luciferase activity

3
2.5
2
1.5
1
0.5
0

control   *Ahr*   *Arnt*   *Arnt2*   *Sim1*

B

■ DMSO   ☐ TCDD

relative luciferase activity

2,5
2
1,5
1
0,5
0

A2   A2m
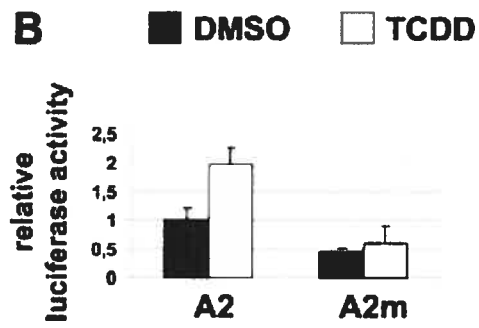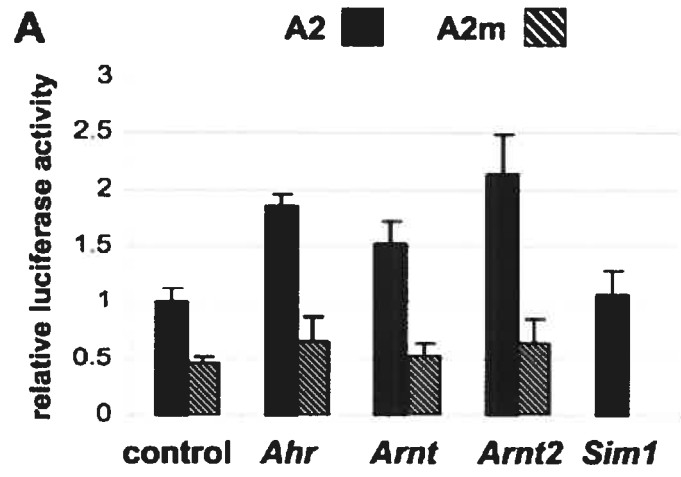
Figure 4.

Figure 4. Effect of *Ah*r, *Arnt* and *Arnt2* overexpression or of TCDD supplementation on *Sim1* promoter activity.

A, N2A cells were transiently cotransfected with an *Ahr, Arnt, Arnt2,*or *Sim1* expression vector and with an expression vector in which the A2 fragment or its mutated version was cloned upstream of the luciferase reporter gene. Relative luciferase activities are expressed as -fold induction over the value obtained from transfection of pGL3-basic empty vector. Results represent the mean ± S.D. of three independent experiments, each performed in duplicate. *B,* N2A cells were transiently transfected with the pGL3-basic plasmid containing the A2 fragment or its mutated version cloned upstream of the luciferase reporter gene in the presence of TCDD or dimethyl sulfoxide (*DMSO*). Relative luciferase activities are expressed as -fold induction over the value obtained from transfection of the nonmutated vector in the presence of dimethyl sulfoxide, which was given an arbitrary value of 1. Results represent the mean ± S.D. of three independent experiments, each performed in duplicate.
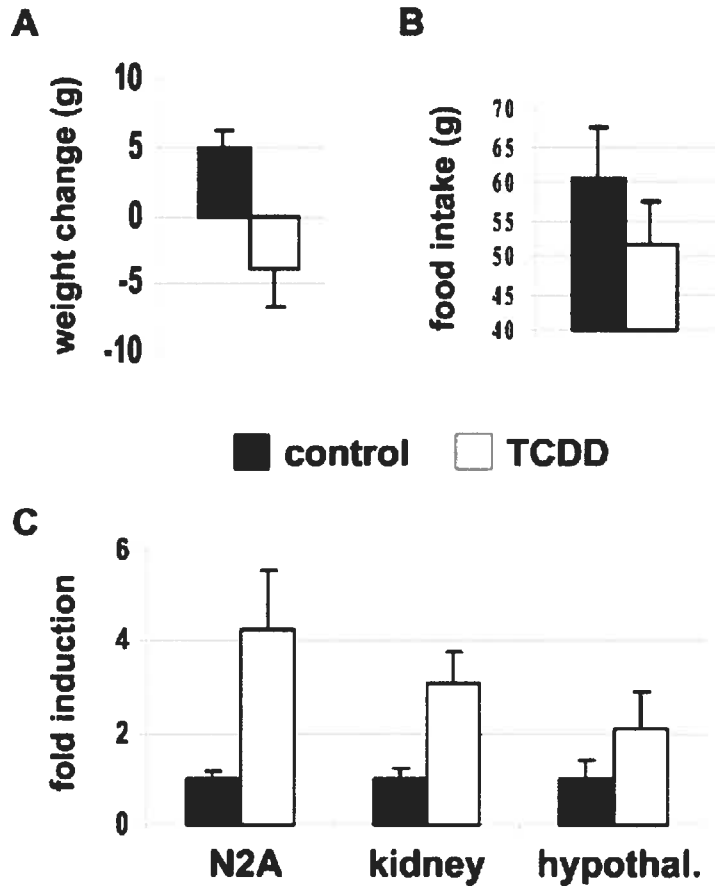
Figure 5.

Figure 5. Effect of TCDD on weight gain, appetite, and *Sim1* mRNA levels.
A and *B*, effect of TCDD administration on weight gain and appetite. Change in weight (in grams) (*A*) and in total food intake (*B*) of mice over a 2-week period following a single dose of TCDD (360 mg/kg) or of vehicle (*n* = 4 for each group). TCDD administration resulted in a significant decrease in weight (*p* < 0.005) and food intake (*p* < 0.05). Statistical analysis was performed with a Student *t* test. *C, Sim1* expression levels in N2A cells incubated with TCDD or dimethyl sulfoxide and in mice kidney and hypothalamus after administration of TCDD or of vehicle expression levels were measured using real-time PCR. Results are expressed as -fold induction using the formula of Buckhaults *et al.* (35) after normalization to β-actin levels and represent the mean of independent reactions performed on three N2A cultures and 12 kidney and eight hypothalamus samples. Each of these tissue samples was collected from a different mouse.

CHAPTER III: ADENOVIRAL-MEDIATED MODULATION OF SIM1 EXPRESSION IN THE PARAVENTRICULAR NUCLEUS AFFECTS FOOD INTAKE

ADENOVIRAL-MEDIATED MODULATION OF SIM1 EXPRESSION IN THE PARAVENTRICULAR NUCLEUS AFFECTS FOOD INTAKE

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ABSTRACT

Haploinsufficency of *Sim1*, which codes for a bHLH-PAS transcription factor, causes hyperphagia in mice and humans, without decrease of energy expenditure. *Sim1* is expressed in several areas of the brain, including the developing and postnatal paraventricular nucleus (PVN), a region of the hypothalamus that regulates food intake. We have previously found that the number of PVN cells is decreased in $Sim1^{+/-}$ mice, suggesting that their hyperphagia is caused by a developmental mechanism. However, the possibility that *Sim1* functions in the postnatal PVN to regulate food intake cannot be ruled out. In order to explore this hypothesis, we used adenoviral vectors to modulate *Sim1* expression in the postnatal PVN of wild-type mice. Unilateral stereotaxic injection into the PVN of an adenoviral vector producing a short hairpin RNA directed against *Sim1* resulted in a significant increase of food intake, which peaked to 22% six days after the procedure, when compared to the injection of a control virus. In contrast, injection of an adenovirus that expresses *Sim1* induced a decrease of food intake that was maximal on the 7th day after the procedure, reaching 20%. The impact of bilateral injections of these vectors into the PVN was not greater than that of unilateral injections. All together, these results strongly suggest that *Sim1* functions physiologically to regulate food intake. Moreover, they suggest the existence of a pathway in the PVN that regulates food intake independently of energy expenditures.

INTRODUCTION

The bHLH-PAS transcription factor SIM1 is required for the development of the paraventricular nucleus (PVN) of the hypothalamus (Michaud et al., 1998). In the absence of *Sim1*, virtually all cells of the PVN are missing and mice die shortly after birth, presumably because of these defects. In contrast, mice with only one copy of a *Sim1* mutant allele survive but show increased weight gain after 4 weeks of age (Michaud et al., 2001) (Holder, Jr. et al., 2004). Oxygen consumption and pair-feeding studies suggest that *Sim1* haploinsufficiency induces obesity by increasing food intake, without decreasing energy expenditure ((Michaud et al., 2001) (Holder, Jr. et al., 2004) (Duplan et al., unpublished observations). Decrease of SIM1 also causes obesity in humans, presumably by inducing hyperphagia (Holder Jr et al., 2000).

The PVN is a critical regulator of both feeding and energy expenditure, acting as a component of a circuit that links the arcuate nucleus, in which leptin functions, and preganglionic neurons of the autonomic nervous system located in the brainstem and in the spinal cord (Swanson and Sawchenko, 1983). Thus, it is plausible that *Sim1* haploinsufficiency causes hyperphagia by interfering with the function of the PVN. Indeed, we have found that the number of PVN cells is decreased by 24% in *Sim1* heterozygous mice, raising the possibility that their hyperphagia has a developmental origin (Michaud et al., 2001). However, since *Sim1* is expressed continuously in the postnatal PVN, it could also function physiologically to regulate food intake (Michaud et al., 2001). Alternatively, *Sim1* could regulate food intake by acting in other regions of the developing and adult brain in which it is expressed, such as the nucleus of the lateral olfactory tract of the amygdala and the ventrolateral hypothalamus (Balthasar et al., 2005). In order to determine by which of these mechanisms *Sim1* haploinsufficiency increases food intake, we used adenoviral vectors to alter *Sim1* expression levels in the PVN of wild-type mice.

MATERIAL AND METHODS

Production of adenoviruses

Three defective serotype 5 adenoviruses (Ad-shRNA-*Sim1*, Ad-*Sim1* and Ad-CTRL) were generated through homologous recombination between PS-expressing shuttle vectors and PS-deleted Ad5 genome cleaved in E1, as previously described (Elahi et al., 2002; Ogorelkova et al., 2004). Construction of shuttle vectors was performed as follows:

1) For production of the Ad-shRNA-*Sim1* virus, we first identified, using the Oligoengine software (Seattle, Washington), two sequences of 19 nucleotides within the *Sim1* coding region of the mouse to design templates for the production of short hairpin RNAs. Briefly, an oligonucleotide containing either one of these 19-nt sequences separated by a short spacer from the reverse complement of the same sequence and a five thymidine termination signal was cloned in the pSUPER expression vector system downstream of an H1 RNA polymerase III promoter (Oligoengine, Seattle, Washington). The efficacy of these constructs in reducing *Sim1* transcript was tested in cultured cell systems. The region containing the H1 promoter and the shRNA template that was the most efficient (5'-CTCGGCTCTCATCTAC TCC-3') was then amplified by PCR from the pSUPER vector and cloned into the shuttle vector pAdCMV5/PS (Gagnon et al., in preparation). This vector also contains, downstream of the shRNA template, a RFP-producing cassette under the control of a human CMV–immediate early promoter.

2) For production of the Ad-*Sim1* virus, a mouse *Sim1* cDNA, which was modified to contain a Kozak translation initiation sequence, was cloned into the pAdCMV5-IRES-GFP/PS vector which co-expresses *Sim1* and *Gfp* from the human CMV-immediate early promoter using an internal ribosome entry site (Gagnon et al., in preparation).

3) A pAdCMV5-RFP/PS vector, which produces RFP under the control of the CMV promoter, was used to generate the control virus (Ad-CTRL) (Gagnon et al., in preparation).

For production of adenoviruses, 10^6 293A cells cultured in a 60-mm dish were transfected with 3 μg of linearized shuttle vector using lipofectin (Life Technologies, Carlsbad, California). Immediately after transfection, Ad5-ΔPS virus at a MOI of 10^6 pfu was added to each dish. Cells were lyzed three days later through cycles of freezing and thawing and viral particles were separated from cell debris by centrifugation. Positive viral recombinants were identified by visualization of GFP or RFP production in 293A cells. Amplification of individual viral clones, their purification with the Adenopure system (Puresyn, Malvern, Pensylvania) as well as determination of viral titer by plaque assay were performed as previously described (Ogorelkova et al., 2004). The titers of the Ad-CTRL, Ad-*Sim1* and Ad-shRNA-*Sim1* stocks were 2.1×10^{12}, 1.8×10^{12} and 2×10^{12} pfu/ml, respectively.

Protein analysis

293A cells were seeded at a density of 1×10^6 cells/60-mm dish and were infected at variable MOI. Cells were harvested 3 days after infection. For Western blot analysis, 20 μg of total protein extract were loaded in each well of a 10% SDS-polyacrylamide gel. Goat SIM1 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California) and mouse actin monoclonal antibody (Abcam Inc., Cambridge, Massachusetts) were diluted to 2 μg/ml and 1:10,000, respectively. Secondary antibodies conjugated with peroxidase were visualized with the 3,3'-diaminobenzidine solution. Signal intensity of the bands was measured with the Fluorchem system (Alpha Innotech Corporation, San Leandro, California).

Stereotaxic injections

Four or five week old CD1 males, weighing between 25 and 30 grams, were housed individually in cages from at least 5 days before surgical interventions

until sacrifice. Each mouse injected with the Ad-shRNA-*Sim1* or the Ad-*Sim1* viruses was paired with a littermate that was injected on the same day with the Ad-CTRL virus. Mice used for the set of unilateral injections with the Ad-shRNA-*Sim1* and Ad-CTRL viruses were maintained on a Harlan Teklad mouse breeder diet (10 % crude fat; Harland Teklad, Madison, Wisconsin) whereas mice used for the other injections were maintained on the Teklad global 19% protein rodent diet (9% crude fat; Harland Teklad, Madison, Wisconsin).

For injections, mice were anesthetised with 100 mg/kg of ketamine (Ayerst, Guelph, Ontario) and 10 mg/kg of xylazine (Ayerst, Guelph, Ontario) injected intraperitoneally. The skull was immobilized in a stereotaxic apparatus (David Kopf Instruments, Tujunga, California). A hole of 1.5 mm was created in the cranium by circular movements of hand held Dumont 5-45 tweezers (Fine Science Tools, Vancouver, British Columbia). The stereotaxic coordinates for injection into the PVN were: 0.85 mm caudal to bregma, 0.15 mm lateral to the midline, and 5.5 mm below the surface of the skull. Using a 31G needle (Hamilton, Reno, Nevada) with a 5 µl Hamilton microsyringe, 0.5 µl of 4 x 10^7pfu/ml or of 1.2 x 10^7 pfu/ml Ad5 vector preparations were injected into the PVN over a 10 minute period. After vector delivery, the needle was left in place for 20 minutes to prevent reflux, and then slowly withdrawn in several steps, over 5 minutes. Food intake and body weight were measured daily from the 3rd or the 4th to the 8th day after the procedure.

Validation of injection site

Eight days after adenoviral injection, mice were anesthetized and perfused with paraformaldehyde 4%, as previously described (Michaud et al., 2001). Brains were removed, post-fixed overnight in paraformaldehyde 4%, cryoprotected with 30% sucrose, embedded in OCT (Sakura Finetek, Torrance, California) and sectioned coronally at 20 µM with a cryostat. Successful injection was defined by the presence of RFP or GFP-producing

cells in the PVN. In situ hybridization using a *Sim1* probe labelled by UTP-S[35] incorporation was performed, as previously described (Michaud et al., 1998). *Sim1* expression in the injected and non-injected PVNs was compared by quantifying hybridization signals using the Image Pro software (Media Cybernetics, Silver Spring, Maryland) on every fourth section. For each mouse, the sum of arbitrary values for the total intensity of signals captured on one side of the PVN was calculated and compared to the sum of intensity values observed on the other side.

Statistical analysis

Results were expressed as means ± SEM. Statistical significance was determined using paired Student's t-test.

RESULTS

In order to modulate *Sim1* expression levels in the PVN, we first generated three adenoviral vectors: Ad-shRNA-*Sim1*, which produces a shRNA directed against *Sim1*; Ad-*Sim1*, which expresses *Sim1;* and Ad-CTRL. We found that infection of 293A cells with the Ad-*Sim1* virus resulted in the abundant production of a SIM1 protein of the expected size (Fig. 1A). Co-infection of cells with Ad-*Sim1* and Ad-shRNA-*Sim1* resulted in a 60% decrease in the production of SIM1, whereas co-infection with Ad-*Sim1* and Ad-CTRL did not affect the production of the SIM1 protein by the former virus (Fig. 1B,C). The CMV promoter used to direct *Sim1* expression is much stronger than the H1 promoter used to direct shRNA expression. This difference may explain why we needed 20 times more Ad-shRNA-*Sim1* than Ad-*Sim1* viral particles to achieve this 60% reduction.

We next used a protocol of stereotaxic injection to infect the PVN of mice with these adenoviruses. In a first series of experiments, we injected 20×10^6 Ad-shRNA-*Sim1* viral particles unilaterally into the PVN of 10 wild-type CD1 mice. Each of these mice was paired with a littermate that was injected on the same day with the same dose of the Ad-CTRL virus. After 4 days of recuperation from surgery, food intake and body weight were measured daily until the end of the 8th day when mice were sacrificed. Two pairs of mice were excluded from the analysis because RFP-producing cells were not detected in the PVN of at least one of the littermates. Food intake was significantly increased from the 5th to the 8th day in mice injected with the Ad-shRNA-*Sim1* virus compared to those injected with the Ad-CTRL virus ($P < 0.005$; n = 8 mice for each virus) (Fig. 2A). The peak of the adenoviral effect was reached on the 6th day when food intake was increased by an average of 22 % in mice injected with the Ad-shRNA-*Sim1* virus. The time course of the effect of Ad-shRNA-*Sim1* on food intake correlated with the fact that Ad5 transgene expression in mouse brain usually peaks from 4 to 7 days postinfection and

declines thereafter (Alisky and Davidson, 2004). There was no significant difference between the body weight of mice injected with the Ad-shRNA-*Sim1* or the Ad-CTRL virus before and after the injections (not shown).

Sim1 expression levels in the infected and non-infected halves of the PVN were compared by in situ hybridization in these mice 8 days after the injection. *Sim1* expression was decreased by 29 %, on average, in the PVN halves infected with the Ad-shRNA-*Sim1* virus (P < 0.005; n = 8 mice for each side), whereas it was not significantly changed in PVNs infected with the control virus (Fig. 2B-E). *Sim1* expression in the PVN halves infected with Ad-shRNA-*Sim1* was decreased by more than 20% in 63 % of sections, indicating that only a subset of the PVN was infected. Histological examination did not reveal grossly abnormal cell density within the infected PVN (Fig. 2B). Of note, injection of 6.0 x 10^6 Ad-shRNA-*Sim1* particles did not affect food intake when compared to the injection of the same dose of the Ad-CTRL virus (data not shown; n = 4 mice for each virus). The initial dose of 20 x 10^6 viral particles was thus used for the remaining experiments.

Using the same protocol of stereotaxic injection, we next compared the effect of the Ad-*Sim1* and Ad-CTRL viruses on food intake. Nine pairs of mice were injected but 2 were excluded from the analysis because GFP- or RFP-producing cells were not detectable in the PVN of at least one littermate. Food intake was decreased from the 6th to the 8th day following the injection in mice infected with the Ad-*Sim1* virus when compared to those infected with the Ad-CTRL virus (P <0.005; n = 7 mice for each virus) (Fig. 3A). The peak of the adenoviral effect was reached on the 7th day when food intake was decreased by 20 %, on average, in mice injected with the Ad-*Sim1* virus. Of note, food intake of mice infected with Ad-CTRL increased slightly but not significantly following the injection. We observed this effect in another set of experiments (see below; Fig. 4) but not in the first one (Fig. 2). There was no significant difference between the body weight of mice injected with the Ad-*Sim1* or the

Ad-CTRL before and after the injections (not shown). In situ hybridization studies indicated that *Sim1* expression was increased on average by 19 % in the infected half of the PVN of mice which received the Ad-*Sim1* virus, but it was not changed in those which received the control virus (Fig. 3B-E). *Sim1* expression in the PVN halves infected with Ad-*Sim1* was increased by more than 20 % in 57 % of sections, suggesting again that only a subset of the PVN was infected.

In order to explore the impact of more extensive changes in *Sim1* expression levels on food intake, we performed a last set of experiments in which both sides of the PVN were injected either with the Ad-shRNA-*Sim1*, Ad-*Sim1* or Ad-CTRL virus on the same day in trios of littermates. RFP- or GFP-producing cells were detectable in both sides of the PVN in all of the injected mice. Bilateral injection of the Ad-shRNA-*Sim1* virus into the PVN significantly increased food intake from the 5th to the 8th day following the procedure (P <0.005; n = 7 mice for each virus), whereas injection of the Ad-*Sim1* virus significantly decreased food intake from the 5th to the 8th day (P < 0.05; n = 7 mice for each virus) (Fig. 4). The most dramatic changes were observed on the 6th day with the Ad-shRNA-*Sim1* virus increasing food intake by an average of 21 % and the Ad-*Sim1* virus decreasing food intake by an average of 17 %, when compared to the effect of the Ad-CTRL virus. Although bilateral infection of the PVN by these viruses did not have a more pronounced effect than unilateral infection, this third set of injections further indicates that modulation of *Sim1* expression levels specifically affects food intake.

DISCUSSION

We have previously reported that *Sim1* is required for the development of the PVN (Michaud et al., 1998). The experiments reported here indicate that *Sim1* plays a second role in the PVN, acting along a physiological pathway to regulate food intake. We cannot, however, exclude the possibility that the structural defects identified in the PVN of *Sim1*^{+/-} mice may also contribute to their hyperphagia. For instance, the obesity of other models, such as leptin deficient mice, may be explained by a combination of developmental and physiological defects (Bouret et al., 2004). The use of a conditional mutant allele of *Sim1* would be necessary to dissect the respective contributions of such factors to the obesity caused by *Sim1* haploinsufficiency.

Sim1 gene copy number has a major influence on food intake in mice and in humans (Holder Jr et al., 2000) (Michaud et al., 2001) (Holder, Jr. et al., 2004). Our paradigm of stereotaxic injection of adenoviruses into the PVN does not easily permit correlations between physiological endpoints and levels of gene expression, since only a subset of the PVN is infected. However, it is noteworthy that reduction of *Sim1* expression only in a subset of the PVN is sufficient to affect food intake. Of note, the impact of bilateral infection of the PVN was not greater than that of unilateral infection. Several explanations could be proposed to account for this observation. For instance, a component of the *Sim1* pathway could be found in limiting amounts in PVN cells, restricting the impact of an increase of *Sim1* expression levels. Also, the impact on food intake of small changes in the activity of the *Sim1* pathway could reach a plateau either because of the intrinsic properties of the pathway or because of the existence of counter-regulatory loops.

Our observation that *Sim1* functions in the PVN to influence food intake should be interpreted in the context of a great body of work establishing this nucleus as a critical regulator of energy balance. First, selective destruction of

the PVN increases food intake (Choi and Dallman, 1999). Second, injection of a variety of anorexigenic and orexigenic compounds into the PVN influences food intake (Cowley et al., 1999). Third, PVN neurons can sense signals provided by axons from the arcuate nucleus on which leptin acts for the regulation of energy balance (Cowley et al., 1999). The PVN appears to integrate several hypothalamic and extra-hypothalamic signals to provide a major hypothalamic output for the regulation of energy intake through its projections to the brainstem (Blevins et al., 2004) (Rinaman et al., 2005). *Sim1* is also expressed in cells scattered in the anteroventral hypothalamus as well as in the nucleus of the lateral olfactory tract (Balthasar et al., 2005). These cells have not been formally associated with the regulation of food intake. Although we cannot exclude the possibility that *Sim1* also functions in these cells, it appears likely that the PVN is its main site of action for the regulation of food intake.

The regulation of food intake and energy expenditure by the PVN appears to involve divergent pathways. For instance, the NPY pathway harboured by the PVN influences feeding, at least in part, through projections to the nucleus of the solitary tract, located in the brainstem, whereas its effects on thermogenesis in the brown adipose tissue has been suggested to involve another neural site (Kotz et al., 1998). Moreover, a recent study using a conditional allele of *Mc4r*, which codes for a melanocortin receptor, strongly suggests the existence of divergent melanocortin pathways: the melanocortin pathway in the PVN controls food intake whereas a melanocortin pathway located elsewhere regulates energy expenditure (Balthasar et al., 2005). All together, our results further support the existence of a specific pathway in the PVN that regulates food intake independently of energy expenditures. Interestingly, mice with a decrease of *Sim1* or of *Mc4r* show phenotypic similarities, both with early-onset hyperphagia and increased linear growth. It is tempting to speculate that *Sim1* regulates a component of the melacortin pathway located in the PVN.

SIM1 can be added to a growing list of PAS domain proteins that are involved in the regulation of physiological processes acting at the interface between the environment and the internal milieu (Gu et al., 2000). Examples of such regulators include Clock and NPAS, which control the circadian rhythm, PAS kinase which modulates insulin expression and HIF which mediates the response of the organism to hypoxia. Interestingly, Clock also functions in the hypothalamus to regulate food intake by a mechanism being characterized (Turek et al., 2005). The activity of some PAS proteins, such as AHR and NPAS, has been shown to be regulated by small ligands (Gu et al., 2000) (Dioum et al., 2002). It is tempting to speculate that *Sim1* is regulated by a similar mechanism, representing an interesting target for pharmacological manipulation.

ACKNOWLEDGEMENTS

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A   Ad-CTRL Ad-*Sim1*

SIM1

actin

B   MOI   5   5   5   Ad-*Sim1*
              0   10   100   Ad-shRNA-*Sim1*

SIM1

actin

C   MOI   5   5   5   Ad-*Sim1*
              0   10   100   Ad-CTRL

SIM1

actin
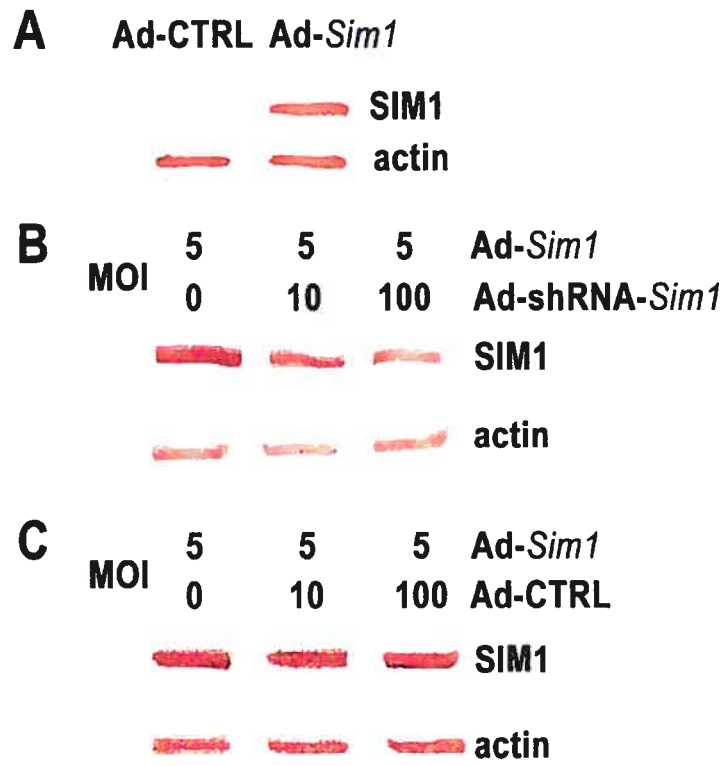
Figure 1.

Figure 1. Western blot detection of SIM1 production in cultured 293A cells infected with adenoviruses. (A) Production of SIM1 protein was detectable in cells infected with Ad-*Sim1* but not with Ad-CTRL. (B) Cells were infected with Ad-*Sim1* (at a constant MOI of 5) and/or Ad-shRNA-*Sim1* (at MOI of 10 or 100). Co-infection with Ad-*Sim1* and Ad-shRNA-*Sim1* resulted in a 40 % decrease of SIM1 production when a MOI of 10 of the latter virus was used, whereas infection with Ad-shRNA-*Sim1* at a MOI of 100 decreased SIM1 production by 60%. Infection by these viruses did not affect actin production. (C) Cells were infected with Ad-*Sim1* (at a constant MOI of 5) and/or Ad-CTRL (at MOI of 10 or 100). Infection with Ad-CTRL did not affect the production of SIM1 protein by the Ad-*Sim1* virus.
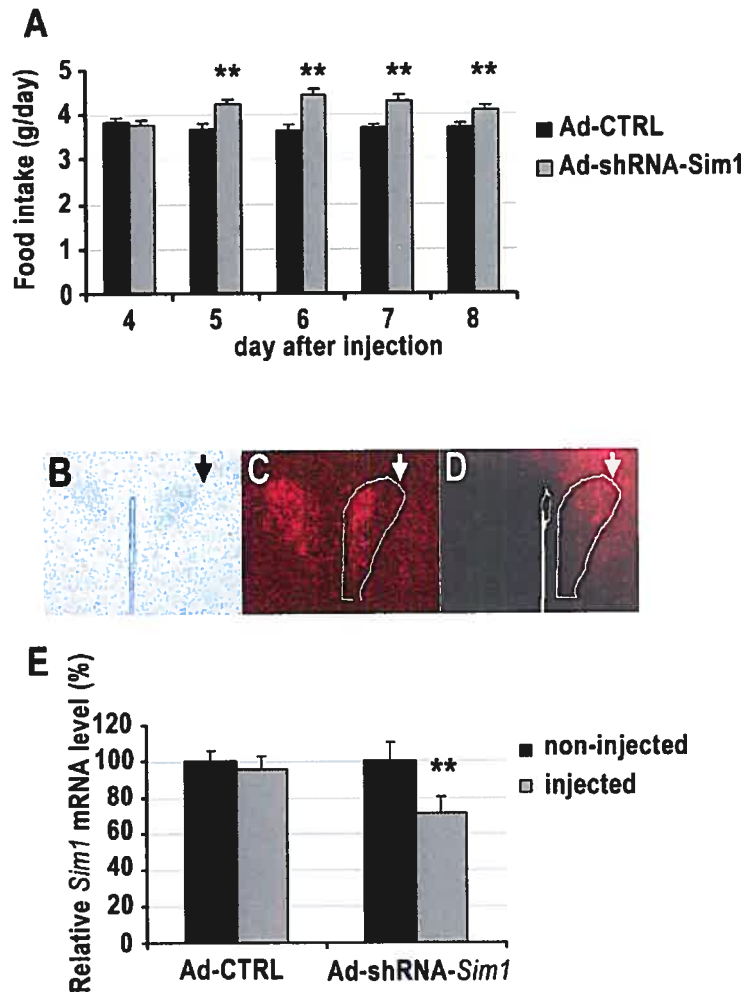
Figure 2.

Figure 2. Unilateral infection of the PVN with the Ad-shRNA-*Sim1* virus increases food intake. (A) Daily food intake of mice infected with Ad-CTRL or Ad-shRNA-*Sim1* (**: P <0.005; n = 8 mice for each virus). **(B,C,D)** Representative coronal sections through the PVN of a mouse infected with Ad-shRNA-*Sim1*. The virus was injected into the right side of the PVN and the mouse was sacrificed 8 days after the infection. **(B)** and **(C)** are brightfield and darkfield views, respectively, of the same section, which was hybridized with a *Sim1* probe, whereas **(D)** is adjacent to **(B)** and **(C)** and shows RFP production. The arrow shown in **(B,C,D)** is positioned on the tract of the needle with its pointed extremity corresponding to the deepest end of the tract. **(E)** Quantification of *Sim1* expression by in situ hybridization. Every fourth coronal section through the PVN of mice infected with the Ad-CTRL or the Ad-shRNA-*Sim1* was hybridized with the *Sim1* probe. The virus was always injected on the right side. The sum of intensity values for each side of the PVN was compared. The values corresponding to the injected side are expressed relatively to those corresponding to the non-injected side, which are set at 100% (**: P < 0.005; n = 8 mice for each side).

A

Food intake (g/day)

■ Ad-CTRL
▨ Ad-*Sim1*

** ** **

day after injection

B **C** **D**

E

Relative *Sim1* mRNA level (%)

■ non-injected
□ injected

*
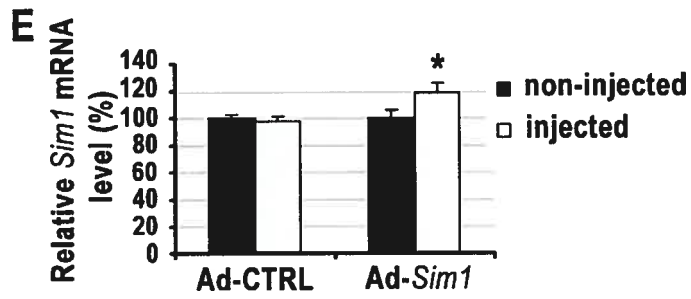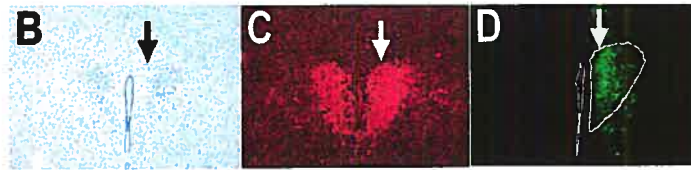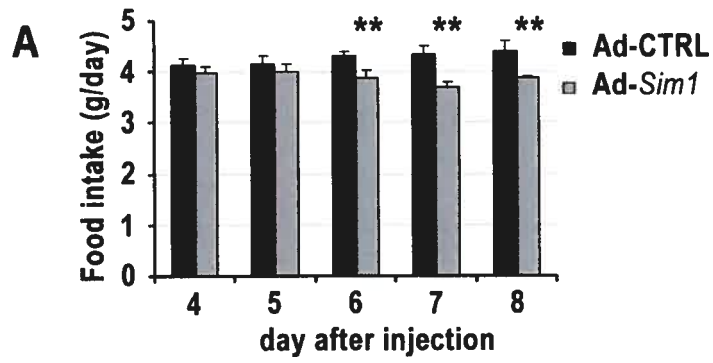
Ad-CTRL   Ad-*Sim1*

Figure 3.

Figure 3. Unilateral infection of the PVN with the Ad-*Sim1* virus decreases food intake. (A) Daily food intake of mice infected with the Ad-CRTL or the Ad-*Sim1* virus (**: P <0.005; n = 7 mice for each virus). **(B,C,D)** Representative coronal sections through the PVN of a mouse which was infected with the Ad-*Sim1*. The virus was injected into the right side of the PVN and the mouse was sacrificed 8 days after the infection. **(B)** and **(C)** are brightfield and darkfield views, respectively, of the same section, which was hybridized with a *Sim1* probe, whereas **(D)** is adjacent to **(B)** and **(C)** and shows GFP production. The arrow shown in **(C)** and **(D)** is positioned on the tract of the needle with its pointed extremity corresponding to the deepest end of the tract. **(E)** Quantification of *Sim1* expression by in situ hybridization. Every fourth coronal section through the PVN of mice infected with the Ad-CTRL or the Ad-*Sim1* was hybridized with the *Sim1* probe. The virus was always injected on the right side. The sum of intensity values for each side of the PVN was compared. The values corresponding to the injected side are expressed relatively to those corresponding to the non-injected side, which are set at 100%. (**: P < 0.005; n = 8 mice for each side).
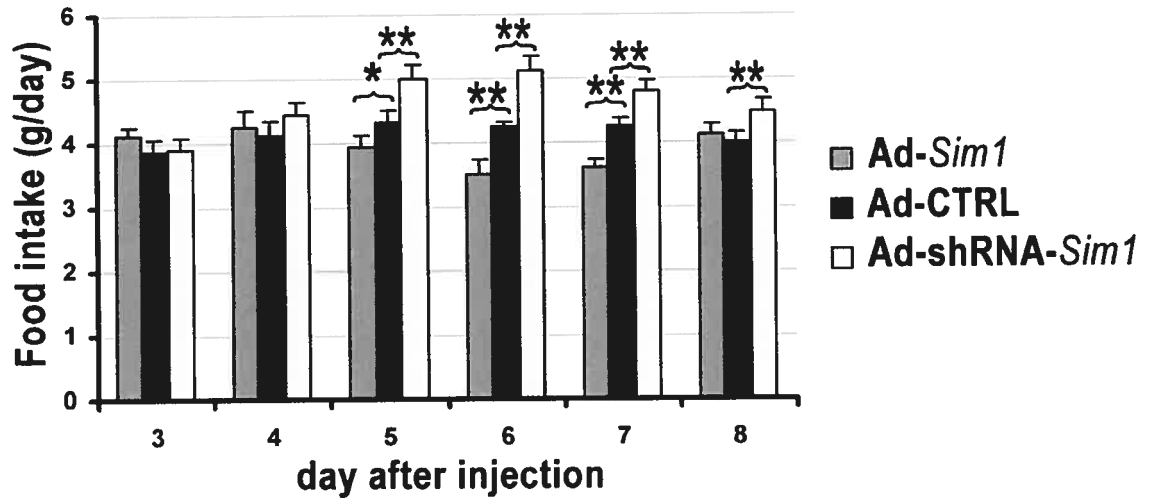
Figure 4. Bilateral infection of the PVN with adenoviruses that modulate *Sim1* expression levels affects food intake. Daily food intake of mice infected with the Ad-*Sim1*, Ad-shRNA-*Sim1* or the Ad-CTRL virus. Mice infected with the Ad-*Sim1* show decreased food intake from the 5th to the 7th day after the injection when compared to mice infected with Ad-CTRL (*: P < 0.05 and **: P <0.005; n = 7 mice for each virus). In contrast, mice infected with Ad-shRNA-*Sim1* show increased food intake from the 5th to the 8th day after the injection when compared to mice infected with Ad-CTRL (**: P <0.005; n = 7 mice for each virus).

CHAPTER IV: GENERAL DISCUSSION

Sim1 haploinsufficiency causes hyperphagia and obesity in mice and human (Michaud et al., 2001; Holder et al., 2000; Holder et al., 2004). Our primary hypothesis was that *Sim1* haploinsufficency increases food intake by inducing some PVN dysfunction. Indeed, it was found that the number of PVN cells is decreased by 24% in *Sim1*^{+/-} mice, raising the possibility that their hyperphagia has a developmental origin (Michaud et al., 2001). However, we cannot exclude the possibility that *Sim1* regulates food intake by acting in the postnatal PVN in which *Sim1* and its partner *Arnt2* are expressed. The main objective of this work was to explore the possibility that *Sim1* function physiologically for the regulation of food intake. We tried to identify a PVN specific regulatory element that would allow us to modulate *Sim1* postnatal function in transgenic mice models; understanding the regulation of *Sim1* gene expression would also allow us to identify upstream components of an important regulatory pathway for the food intake. Although we found that *Sim1* expression is regulated by the bHLH-PAS complex AHR-ARNT2, we did not identify such a PVN element. In order to directly alter *Sim1* expression levels in the PVN of adult mice, we used, as an alterative approach, the stereotaxic microinjection of adenoviruses that produce the SIM1 protein or a shRNA direct against *Sim1*. These experiments strongly suggest that *Sim1* is a regulator of food intake.

1. Characterization of regulatory elements driving *Sim1* expression in the brain

Sim1 is strongly expressed throughout the PVN during development and after birth (Fan et al., 1996). The study of *Sim1* regulation has the potential of leading to the identification of a specific PVN element that could be used for functional studies in transgenic experiments. We first characterized *Sim1* transcriptional initiation site by 5'-RACE analysis using mRNA purified from murine embryonic brains obtained from 12.5 days post-coitus and newborn hypothalamus (Michaud et al., 1998). We found that the transcription initiation site (TSS) is located 1121 nucleotide upstream of the translation start

site. This result is supported by the computer prediction program FirstEF (First Exon Finder), which is a 5' terminal exon and promoter prediction program (Ramana et al., 2001), and is further confirmed by BLASTN searches of an ESTs (Expressed Sequence Tags) database sequences which revealed the existence of an EST containing the same extended 5' transcribed region. Moreover, our transfection studies revealed the presence of a *Sim1* presumptive basal promoter located just upstream of the TSS.

Taking advantage of this information, we characterized an 8.1 kb 5' sequence, that contains the *Sim1* basal promoter and a conserved region located in the second intron of the *Sim1* gene (annexe 2). All together, results from in vitro and in vivo experiments suggest that: 1) the *Sim1* promoter activity is down regulated by repressor elements located upstream in the 5'-sequence analyzed; 2) the combination of two conserved regions (M+A) is able to drive reporter gene expressing in a caudal hypothalamic domain overlapping with a *Sim1* domain; 3) elements required for *Sim1* expression in the PVN are not located in the 8.1kb 5'-region that we have characterised. In order to further characterise PVN specific elements, the next approach would be to analyse a *Sim1* bacterial artificial chromosome (BAC) in transgenic mouse lines. A *Sim1* BAC (RPC123-20F11), which contains 137.1kb of genomic 5'-*Sim1* sequences, has been shown be able to drive reporter gene expression in all *Sim1* domains (Balthasar et al., 2005; Heintz, 2004). Taking advantage of an *E coli*-based chromosome engineering system, we could "knock-in" the LacZ or GFP reporter gene into this *Sim1* BAC, and generate different transgenic constructs with any region of the *Sim1* BAC (Lee et al., 2001). In this way, we would be able to identify regulatory elements that may be located at some distance from the gene.

2. Interaction between SIM1 and AHR

In order to advance from the analysis and manipulation of gene expression towards a general understanding of complex gene interactions and epistatic relationships, it is necessary to gain insight into the *cis*-regulation of the *Sim1*

gene. Functional deletion studies of the fragment N demonstrated the existence of a positive regulatory sequence in a small region between 173nt and 220nt upstream of TSS. This region contains a potential binding site (GCGTG) for the bHLH-PAS heterodimer AHR-ARNT2. In order to address the biological function of this site, we performed electrophoretic mobility shift assay, mutagensis, and cotransfection experiments. All together, our results suggested that AHR-ARNT2 and TCDD, a ligand of AHR, are able to increase the promoter activity of fragment A in cultured cells system. We have also studied the effect of TCDD on *Sim1* gene expression in N2A cells, as well as in the kidney and hypothalamus of C57BL/6 mice using quantitative real-time PCR. These results strongly suggest that *Sim1* expression is also regulated in vivo by TCDD.

At sublethal concentrations, TCDD induces anorexia and body weight loss as part of the so-called wasting syndrome (Pohjanvirta & Tuomisto, 1994; Seefeld et al., 1984). The exact mechanism by which TCDD affects feeding is unknown but it may involve the hypothalamus in view of the central role of this structure in the control of energy balance. Moreover, expression of *Ahr*, *Arnt* and *Arnt2* has been demonstrated in the rat hypothalamus (Huang et al., 2000, 2003; Petersen et al., 2000). Several lines of evidence in fact suggest that *Ahr* can act in the PVN, (Peterson et al., 2000; Huang et al., 2000 ; Cheng et al., 2002 ; Fetissov et al., 2004). Our finding that TCDD increases *Sim1* transcript levels in the hypothalamus further supports the idea that TCDD can modulate gene expression in this structure. One possibility is that *Sim1* mediates the effect of TCDD on feeding.

Recently, production of bHLH-PAS proteins in the rat hypothalamus by TCDD was studied by the Pohjanvirta's group (Korkalainen et al., 2005). mRNA levels of *Ahrr* and two target genes of TCDD, *Cyp1a1* and *Cyp1a2*, were significantly increased by TCDD treatment, but not those of *Ahr, Arnt* or *Arnt2. Sim1* expression was not found to be altered by TCDD. This finding does not agree with what we observed in our mouse model. One possibility to account for this discrepancy is that *Sim1* may not be regulated by AHR-

ARNT/2 in the rat. Indeed, we have found that the sequence homolog to the mouse AHR-ARNT/2 binding site is different by one nucleotide. The rat sequence is not predicted to bind AHR-ARNT/2. Therefore, results obtained in the rat do not support our hypothesis that *Sim1* is the major mediator of the effect of TCDD on food intake. However, it is possible that it contributes to this effect in mice.

3. Modulation of *Sim1* expression levels in postnatal PVN using adenoviral vectors

3.1. Technical considerations: use of adenovirus to modulate *Sim1* expression

Our analysis of *Sim1* 5'-sequence did not allow us to identify a PVN specific element. In order to modulate *Sim1* expression levels in the PVN of mice, different strategies could be developed. We could use BAC transgenesis to increase *Sim1* expression. However, one problem is that this transgene would drive *Sim1* expression during development as well as after birth. Moreover, its expression would not be restricted to the PVN domain but would also include other domains of expression in the brain. Alternatively, we could use a transgene that is active in most neurons only after birth or upon induction by a small molecule (Utomo et al., 1999). Again, this approach would not allow us to specifically study the involvement of the PVN. The creation of a conditional allele of *Sim1* using the Cre-loxP system of recombination could resolve the issue as long as a cre-transgene specifically active in the postnatal PVN is available. However, the production of such an allele is time-consuming. We decided to use the injection of viral vectors to modulate *Sim1* expression in the PVN. This approach allows one to control the timing and the site of delivery.

In recent years, techniques of gene transfer into the CNS of rats and mice has provided an unique opportunity to study specific neural cells and functional systems (Weihl et al., 1999). Three types of modified viral vectors have been

commonly used to deliver functional and /or reporter genes to the brain: lentiviral, adeno-associated viral (AAV) and adenoviral vectors.

1) Lentiviruses are a type of retrovirus that can infect both dividing and nondividing as well as terminally differentiated cells, such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, because their preintegration complex can get through the intact membrane of the nucleus of the target cell. They can express genes of interest for up to six months. The best developed and characterized lentiviral vector system is based on the human immunodeficiency virus type 1 (HIV-1). Their lack of safety and the difficulty of producing high titers of these viruses are the main concerns (Sinn et al., 2005).

2) AAV gets its name from the observation that it is often found in cells that are simultaneously infected with adenoviruses. Recombinant AAV (rAAV) vectors offer some important advantages for gene therapy because they mediate stable transgene expression in terminally differentiated cells without inducing significant inflammatory toxicity (Flotte & Ferkol, 1997; Muzyczka, 1994). The use of rAAV is somewhat limited by inefficient production methods, and their small size, which only permits the packaging of 4.7 kilobases (kb) of exogenous DNA, including the regulatory elements and 3' UTR sequence (Zolotukhin et al., 1999; Dong et al., 1996).

3) Adenovirus (Ad) offers the following advantages: it is relatively safe; it can be generated in high titers; it can transfect nondividing cells, and can undergo retrograde transport from nerve terminals to somata (Davidson & Bohn, 1997). The Ad vector has been used successfully to deliver genes to the central nervous systems (Davidson et al., 1993; Ghodsi et al., 1998; Kuo et al., 1995; Le Gal La Salle et al., 1993; Ridoux et al., 1994; Vasquez et al., 1998). For all these reasons, we decided to use adenoviral vector for our study. The Ad vectors used in our experiments are protease (PS) deleted adenoviruses. The Ad PS is one of the essential late viral genes involved in many steps of the virus cycle. PS deleted viruses are capable of only one round of replication in non-complementing cells. This feature was exploited to develop a positive

selection method for constructing adenoviral recombinants using ectopic expression of the PS gene in the E1 region; this process usually results in a 100% efficiency of recombinant selection (Elahi et al., 2002). Three adenoviruses which express *Sim1*, RFP or a short hairpin RNA (shRNA) against *Sim1*, were successfully delivered into the PVN region by stereotaxic microinjection. This study has demonstrated the feasibility of using Ad vectors in gene expression studies. However, this approach has the major limitation that the expression of an adenoviral transgene is relatively transient. It has been suggested that the short-lived in vivo expression of such a transgene is most probably related to the induction of a host-specific cytotoxic T lymphocyte (CTL) immune response directed against viral antigens synthesized at low levels in the transduced tissues (Alisky & Davidson, 2004). As a result of this immune response, combined with the shutdown of cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoters, AD5 transgene expression in mouse brain peaks from 4 to 7 days postinfection and declines thereafter (Alisky & Davidson, 2004). Despite the transient nature of adenoviral infection, this approach allowed us to address the question of *Sim1* function in the postnatal PVN.

3.2. Regulation of food intake by *Sim1*

The injection of the Ad-shRNA-*Sim1* was increasing, whereas the injection of Ad-*Sim1* was decreasing food intake. We have also observed that the period of significant changes of food intake are from 5th day to 8th day after injection, correlating with the known kinetics of adenoviral mediated expression (Alisky & Davidson, 2004). These observations strongly suggest that *Sim1* plays a physiological role in the PVN to regulate food intake.

The use of adenoviruses to dissect hypothalamic pathways is a well established paradigm. However, it should be underlined that RNA interference can have toxic effects, inducing nonspecific changes that are sequence dependent (Scacheri et al., 2004). The best control would be to rescue RNA interference by coinfection with an adenovirus that expresses a *Sim1* cDNA from different specie than mice. However, this type of rescue experiment is

more difficult to perform in vivo. As a complementary approach to confirm the results from the shRNA-*Sim1* knock-down experiment, a *Sim1* expression experiment was performed by injection of an Ad-CMV5-*Sim1* virus into the postnatal PVN. It is possible that the adenoviral-mediated expression of *Sim1* interferes with the function of other bHLH-PAS pathways involved in the regulation of food intake, due to the fact that different bHLH-PAS proteins can compete for binding to ARNT/2 or to DNA target sequence (Woods & Whitelaw, 2002). For instance, CLOCK, another bHLH-PAS protein, has recently been found to control food intake. Loss of CLOCK function induces hyperphagia. Increased expression of *Sim1* could thus interfere with CLOCK function. However, the mechanism underlying this function of CLOCK is unknown (Turek et al., 2005). Moreover, it is not clear whether CLOCK can bind ARNT/2 or whether it is expressed in the PVN.

3.3. Diverging pathway controlling energy balance

Recent studies indicate that food intake and energy expenditures are regulated by divergent pathways within the PVN (Kotz et al., 1998). Neuropeptide Y (NPY) injected into the PVN increases feeding and decreases brown adipose tissue (BAT) uncoupling protein (UCP) and lipoprotein lipase (LPL) mRNA. After injection of low dosage of naltrexone (NTX), an opioid antagonist, into the rostral nucleus of the solitary tract (rNST), the feeding response to PVN NPY was significantly and dose dependently decreased, whereas the NPY-induced decrease in BAT UCP or LPL mRNA was not altered. These results indicate possible divergence in the PVN NPY feeding-stimulatory/BAT-inhibitory pathway, such that PVN NPY feeding effects may be routed through the rNST whereas BAT effects may be due to alterations at another neural site. Activation of melanocortin-4-receptors (MC4Rs) reduces body fat stores by decreasing food intake and increasing energy expenditure. MC4Rs are expressed in multiple CNS sites, any of which could mediate these effects. The Lowell group has recently generated a loxP modified, null *Mc4r* allele that can be reactivated by Cre-recombinase and shown that melanocortin decreases food intake by acting specifically in the PVN whereas melanocortin-

mediated regulation of energy expenditure involves other sites (Balthasar et al., 2005). Our results further reinforce this concept that *Sim1* regulates a pathway centred on the PVN that specifically controls food intake.

The hyperphagia phenotype of *Sim1* heterozygous is similar to that of mice with melanocortin 4 receptor (*Mc4R*) mutations. Both mutants significantly increased their acute caloric intake in response to an increase in dietary fat content and show increased linear growth (Michaud et al., 2001; Holder et al., 2004; Huszar et al., 1997). Moreover, *Sim1* and *Mc4r* are both expressed in the PVN, in which they act to regulate a specific pathway for food intake. But *Mc4r* does not appear to be regulated by *Sim1* raising the possibility that other component of the *Mc4R* pathway are regulated by *Sim1* (Holder et al., 2004). The interaction between *Sim1* and *Mc4R* could be investigated by studying the impact of the injection of MTII, a MC4R agonist, on food intake of $Sim1^{+/-}$ mice. The observation that $Sim1^{+/-}$ mice do not respond to MTII would suggest that *Sim1* function along the melanocortin pathway to control food intake. It is difficult to interpret results of experiments aiming at dissecting interactions with other pathways in $Sim1^{+/-}$ mice because of the associated developmental defects. These defects have the potential of interfering with signalling in the PVN and also of causing some remodelling of the brain which can change the impact of these pathways on the regulation of food intake. Mice with a conditional allele of *Sim1* would allow us to study these signalling pathways in the PVN of mutant mice without the developmental defects.

4. Investigation of mechanisms of *Sim1* pathway controlling food intake.
4.1. Mechanisms involved in the feeding behaviour of $Sim1^{+/-}$ mice

Our observations thus point to at least two mechanisms that are not mutually exclusive to account for the hyperphagia of $Sim1^{+/-}$ mice. Decrease of *Sim1* could induce hyperphagia either by interfering with the development of the PVN or by disrupting a physiological pathway harboured by the PVN. Interestingly, ablation of the PVN by stereotaxic surgery or of some specific cell types in the ARC by genetic engineering in the rodent neonate does not

induce hyperphagia, whereas ablation of the same structures in the adult results in severe increase of food intake (Bernardis, 1984; Luquet et al., 2005). These experiments suggest some plasticity of hypothalamic circuitries: remodelling of the brain would compensate for the loss of these specific cell types. It is thus possible that similar compensatory mechanisms exist in $Sim1^{+/-}$ mice, decreasing the impact of their structural defects on food intake. Alternatively, a combination of developmental and physiological anomalies may cause the hyperphagia of $Sim1^{+/-}$ mice. It is noteworthy that disruption of both developmental and physiological processes also underlies the obesity of leptin deficient mice. In addition to its regulatory role in mature animals, leptin acts to promote the growth of ARC axons projecting to the PVN during development (Bouret et al., 2004).

The precise impact of the $Sim1$ physiological pathway for the regulation of food intake and the contribution of the structural defects of $Sim1^{+/-}$ mice to their hyperphagia remains unknown. This question could be addressed, in part, by injecting the $Sim1$-overexpressing adenovirus into the PVN of these mice in an attempt to rescue the phenotype. However, because of the variability of the hyperphagia of these mice and the variable extent of viral infections, this approach would be tedious, requiring a great number of injections. Also, it is possible that some remodelling of the brain affects the response to an increase of $Sim1$ expression levels. A conditional allele would allow us to resolve this issue by making direct comparisons between mice in which $Sim1$ was already inactivated in the germline and mice with a stable decrease of $Sim1$ starting only after birth.

4.2. Development of the hypothalamus and the search for obesity genes

In view of the work done on $Sim1$, other genetic factors involved in PVN development such as Otp, $Arnt2$, and $Brn2$ also represent potential causes of obesity. $Brn2$ has been found to act downstream of $Sim1$ to control the development of the PVN (Michaud et al., 1998; Goshu et al., 2002). In cotransfection assays with reporter constructs containing the 5' regulatory region of $Brn2$, which has a CME site, no detectable transactivation or

repression by either SIM1 or SIM2 in concert with ARNT or ARNT2 was observed, which suggested that *Brn2* may not be a direct target of *Sim1* (Moffet & Pelletier, 2000).

Identification of *Sim1* transcriptional targets will also provide insight into its proposed role for feeding behaviour. Microarray analysis is a cost-effective approach which would allow to directly compare the RNA expression profiles of the PVN of mutant and control mice. However, expression profiling of the PVN of *Sim1*$^{+/-}$ mice would be difficult to interpret, because of the associated developmental defects. The creation of a *Sim1* conditional allele is thus a critical step for the long-term exploration of this important pathway. An alternative would be studying the effect of *Sim1* overexpression in a cell culture system, since PVN hypothalamic transformed neuron cells are available at this moment (Belsham et al., 2004).

4.3. Generation of a conditional allele of *Sim1*

As we mentioned above, conditional disruption of *Sim1* postnatally would be useful for confirming the results obtained with the adenoviral vectors, for dissecting the pathway along which *Sim1* functions in the mature PVN and for investigating whether other *Sim1* expressing regions are also involved in the regulation of food intake.

Exon 2 of *Sim1* gene contains the initiation codon and the sequence coding for the basic and HLH domains. Previous targeting experiments have shown that deletion of this region interferes with the production of a functional protein (Michaud et al., 1998). One strategy to create a conditional allele of *Sim1* would be to introduce loxP sites on both sides of this exon. In the presence of Cre, recombination will occur between these directly repeated loxP sites resulting in deletion of gene sequence between them (Nagy, 2000) (Fig 1).
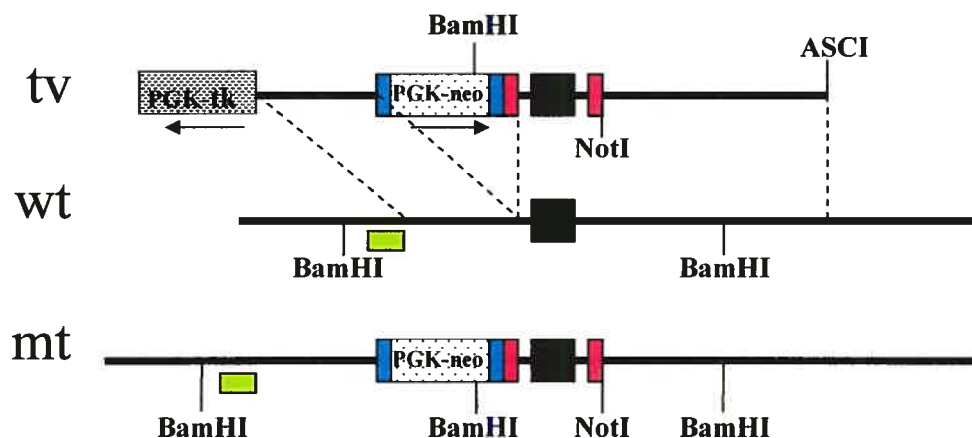
Figure 1. Targeting of the *Sim1* locus. The targeting vector (tv), the *Sim1* wild-type locus (wt) and the targeted locus (mt) are represented. The exon is shown as a black box which is flanked by loxP sites (red boxes). *Pgk-neo* is flanked by frt sites (blue boxes). The vector will be linearized at the AscI site. The 5' external probe indicated by a horizontal green box detects a wild-type 5.2 kb BamHI fragment and a mutant 3.7 kb BamHI fragment.

Cre-trangenes that are active in most neurons after birth have been described. For instance, the neurofilament-H (NF-H) regulatory element has been shown to confer specific and widespread neuronal expression, starting at E18.5 (Hirasawa et al., 2001), and a CamKIIα line has been shown to drive Cre expression in most neurons after P5 including in those of the hypothalamus (Dragatsis & Zeitlin, 2000; Dragatsis et al., 2000). Because a regulatory element that is expressed specifically in PVN neurons has not been described, the Cre-expressing transgenic lines that we mentioned above would lead to the inactivation of *Sim1* in most CNS neurons, which might be difficult to interpret. An alterative approach would be to induce exon2 excision using cre-producing adenoviruse. Such a strategy would result in a more stable decrease of Sim1 expression level with minimal toxic effect, compared to the adenoviral approach we used in the study.

In the adult, *Sim1* is also strongly expressed in the lung, muscle and kidney (Probst et al., 1997). The *Sim1* conditional allele would represent a valuable tool to study its function in these adult tissues. Regulatory elements are available to drive Cre expression in these tissues (Kim et al., 2001; Stricklett et al., 1999).

4.4. Characterization of small molecule regulating *Sim1*

SIM1 belongs to a protein family characterised by the PAS domain, which has a variety of known functions that include ligand and protein-protein interactions (Gu et al., 2000). Some PAS proteins, such as AHR and NPAS have been shown to be activated by specific ligands (Dioum et al., 2002; Gu et al., 2000). It would be interesting to determine whether *Sim1* requires a ligand for proper function. One approach to identify such a ligand would be to test candidate small molecules in a hypothalamic cell culture assay. When activated, SIM1 forms dimers with ARNT/2 and translocates to the nucleus, as it is the case for AHR. It is thus possible that a small molecule activates the translocation of SIM1 into the nucleus. If so, small molecules could be tested for their ability to induce SIM1 translocation in such a system. Monitoring the changes of promoter activities of a *Sim1* direct target gene could be an alternative approach. Small ligands regulatory *Sim1* activity could represent critical pharmacological target for the treatment of obesity.

5. Redundant roles of *Sim1* and *Sim2* in developing MB

Sim1 and *Sim2* are also co-expressed in the developing MB. Our studies strongly suggest that *Sim1* and *Sim2* are required for MB axonal growth and that *Sim1* and *Sim2* function compensatory during MB development (Marion et al., 2005). Since *Sim1* functions in postnatal PVN for controlling food intake, *Sim1* and *Sim2* may similarly be required in the postnatal MB for optimal learning and memory. Postnatal excision of exon 2 from the conditional *Sim1* allele would provide a tool to investigate this possibility. Alternatively, knockdown of *Sim1* expression in postnatal MB by

microinjection of Ad-siRNA-*Sim1* could also represent an approach to explore this possibility.

Conclusion

SIM1 belongs to the family of bHLH-PAS proteins. Interestingly, some other members of this family participate in the regulation of physiological processes. For instance, CLOCK and NPAS regulate the circadian rhythm, whereas HIF mediates the response of the body to hypoxia. We have provided evidence that *Sim1* function in the postnatal PVN to control food intake. It is intriguing that many bHLH-PAS proteins are involved in regulating physiology processes at the interface between the environment and the internal milieu. Also, it would be interesting to determine whether these processes interact though these bHLH-PAS proteins.

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ANNEXE I: SIM1 AND SIM2 ARE REQUIRED FOR CORRECT TARGETING OF MAMMILLARY BODY AXONS

SIM1 AND SIM2 ARE REQUIRED FOR CORRECT TARGETING OF MAMMILLARY BODY AXONS

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Abstract

The mammillary body (MB) and its axonal projections to the thalamus (mammillothalamic tract, MTT) and tegmentum (mammillotegmental tract, MTEG) are components of a circuit involved in spatial learning. The bHLH-PAS transcription factors SIM1 and SIM2 are co-expressed in the developing MB. We have found that MB neurons are generated and survived at least until E18.5 in embryos lacking both $Sim1$ and $Sim2$ ($Sim1^{-/-};Sim2^{-/-}$). However, the MTT and MTEG are histologically absent in $Sim1^{-/-};Sim2^{-/-}$ embryos and reduced in embryos lacking $Sim1$, but bearing one or two copies of $Sim2$, indicating a contribution of the latter to the development of MB axons. We have generated, by homologous recombination, a null allele of $Sim1$ ($Sim1^{tlz}$) in which the tau-$lacZ$ fusion gene was introduced, allowing staining of MB axons. Consistent with the histological studies, $lacZ$ staining showed that the MTT/MTEG is barely detectable in $Sim1^{tlz/tlz};Sim2^{+/-}$ and $Sim1^{tlz/tlz};Sim2^{-/-}$ brains. Instead, MB axons fasciculate and grow towards the midline. $Slit1$ and $Slit2$, which code for secreted molecules that induce repulsion of ROBO1-producing axons, are expressed in the midline at the level of the MB whereas $Robo1$ is expressed in the developing MB. The expression of Rig-$1/Robo3$, a negative regulator of $Slit$ signalling, is upregulated in the prospective MB of $Sim1/Sim2$ double mutants, raising the possibility that the growth of mutant MB axons towards the midline is caused by a decreased sensitivity to SLIT. Finally, we found that $Sim1$ and $Sim2$ act along compensatory, but not hierarchical pathways, suggesting that they play similar roles in vivo.

Introduction

The mammillary bodies (MB), which lie at the caudal end of the hypothalamus, are composed of the lateral and medial mammillary nuclei. The MB participate in two pathways that are essential for processing spatial information (reviewed in (Vann and Aggleton, 2004)). First, The MB link two brain regions that are critical for episodic spatial memory, the hippocampus and the anterior thalamus, by receiving afferents from the former via the fornix, and sending efferents to the latter via the mammillothalamic tract (MTT) (Van der Kooy et al., 1978; Cruce, 1977; Hayakawa and Zyo, 1989; Allen and Hopkins, 1990). Second, the lateral mammillary nucleus contains head direction cells that aid navigation by firing selectively when an animal is facing in a specific direction in the horizontal plane (Vann and Aggleton, 2003). Reciprocal loops connect the MB and some tegmental centers, with the efferent MB axons forming the mammillotegmental tract (MTEG) (Sharp et al., 2001). The lateral mammillary nucleus is probably important for transforming the vestibular information provided by the tegmentum to help signal head direction. It has been suggested that the head direction cells are responsible for the spatial memory function of the MB. However, lesions involving both the lateral and medial nuclei result in more severe learning defect then those restricted to the lateral nuclei, suggesting that the medial nuclei also contribute to spatial memory process.

Most MB neurons send axonal projections to both the anterior thalamic nuclei and the tegmentum via the MTT and the MTEG, respectively. The MTEG is one of the earliest tracts to develop in the CNS, appearing at about E10.5 (Easter, Jr. et al., 1993; Mastick and Easter, Jr., 1996). Much later, at about E17.5, each axon of the MTEG generates collateral that will contribute to the formation of the MTT (Alvarez-Bolado et al., 2000; Valverde et al., 2000). A minority of MB neurons appear to contribute only to the MTT (Hayakawa and Zyo, 1989). MTT axons are induced near the boundary between the dorsal and

ventral thalami. Recent observations indicate that the transcription factors PAX6 and FOXB1 regulate expression of signals in this region that induce and/or guide MTT axons (Valverde et al., 2000; Alvarez-Bolado et al., 2000). Both *Pax6* and *Foxb1* mutant mice are born with an intact MTEG but without a MTT. In *Foxb1* embryos, MTT axons are induced but do not grow into the thalamus whereas branching does not occur at all in *Pax6* mutants. PAX6 is produced in a domain surrounding the MTEG at the level of the bifurcation as well as along the dorsal border of the ventral thalamus. *Foxb1* is expressed along the ventral border of the dorsal thalamus but also in the MB. Chimera analysis, however, indicates that *Foxb1* functions in the thalamus to promote MTT formation. The requirements for MTT axon guidance are thus complex, since the signals controlled by *Pax6* and *Foxb1* are produced by closely located but non-overlapping regions of the thalamus.

The bHLH-PAS transcription factor SIM1 and SIM2 are closely related paralogs which expression overlaps in regions of the anterior hypothalamus that will give rise to the paraventricular (PVN), supraoptic (SON) and anterior periventricular (aPV) nuclei (Fan et al., 1996). *Sim1* is required for the differentiation of virtually all neurons of the PVN/SON/aPV whereas *Sim2* controls the differentiation of a subset of PVN and aPV neurons (Michaud et al., 1998; Goshu et al., 2004). The interplay between *Sim1* and *Sim2* is complex, mutant analysis indicating that *Sim1* acts upstream of *Sim2* but can also compensate for the lack of *Sim2*, albeit ineffectively. SIM1 and SIM2 belong to a group of proteins that need to heterodimerize with members of another group of bHLH-PAS proteins for which there are only four representatives yet characterized: ARNT (Hoffman et al., 1991), ARNT2 (Hirose et al., 1996), BMAL1/MOP3 (Hogenesch et al., 1997; Ikeda and Nomura, 1997; Takahata et al., 1998; Wolting and McGlade, 1998) and BMAL2/MOP9 (Hogenesch et al., 2000; Maemura et al., 2000; Okano et al., 2001; Ikeda et al., 2000). Biochemical, expression and mutant analyses indicate that ARNT2 acts as the dimerizing partner of SIM1, and presumably

SIM2, for anterior hypothalamus development (Michaud et al., 2000; Hosoya et al., 2001; Keith et al., 2001).

Sim1 and *Sim2* are also expressed in the prospective MB. Their function during development of this structure has not yet been elucidated. Here, we show that MB neurons are generated but do not develop MTEG and MTT in embryos lacking both *Sim1* and *Sim2*. Instead, MB axons aberrantly cross the midline. The same abnormalities, although less severe, are observed in embryos with reduced dosage of *Sim1* or *Sim2*. Expression and mutant studies indicate that *Sim1* and *Sim2* act along compensatory pathways that do not required *Arnt2* function. We propose that *Sim1* and *Sim2* regulate the expression of molecules involved in the polarized growth of MB axons.

Material and Methods

Generation of the *Sim1^{tlz}* allele. Two and 4.5 kb fragments, corresponding respectively to the left and right arms of the targeting vector, were amplified by PCR from a *Sim1* BAC clone originating from a 129/Sv mouse genome and cloned into a Bluescript plasmid. A *Pgk-neo* cassette was inserted downstream of a *Tau-lacZ* fusion gene (generous gift of J. Callaghan) and *loxP* sites were introduced on both sides of *Pgk-neo*. The whole *Pgk-neo/Tau-lacZ* cassette was cloned between the two arms of homologous sequences. Finally, a *Pgk-tk* cassette was inserted at the 5' end of the construct.

Twenty micrograms of the construct was linearized at a AscI site located at its 3' end and electroporated into passage 13 R1 ES cells, which were grown as previously described (Michaud et al., 1998). To obtain negative and positive selection for homologous recombinants, gancyclovir and G418 were added to the culture medium at a final concentration of 0.55μg/ml and 150 μg/ml, respectively. Double resistant clones were further analyzed by Southern blot

using a probe containing *Sim1* genomic sequences 5' of those used in the targeting vector. This probe hybridizes to a 5.2-kb BamHI fragment of the wild-type *Sim1* allele and to a 4.5-kb BamHI fragment of the *Sim1* mutant allele. Homologous recombinant ES cell clones were microinjected into C57BL/6 blastocysts to produce chimeric mice. The resulting male chimeras were backcrossed to C57BL/6 females.

Genotyping of mice. Production and genotyping of mice and embryos carrying the *Sim1⁻* or *Sim2⁻* alleles were previously described (Michaud et al., 1998; Goshu et al., 2003). *Sim1ᵗˡᶻ* embryos and mice were genotyped by PCR, using two sets of primers. The first set was designed to detect the mutant allele, and amplifies a 189-bp fragment of the *neo* gene. The second set was designed to detect the wild-type *Sim1* allele, and amplifies a 250-bp fragment that is deleted in the mutant allele. The sequences of these primers are as follows: *neo*, CTCGGCAGGAGCAAGGTGAGATG and GTCAAGA CCGACCTGTCCGGTGC; *Sim1*, CCGAGTGTGATCTCTAATTGA and TAGGCACAGACGCTTACCTT. The reaction was carried out at 94°C for 30 sec., 54°C for 45 sec, and 72°C for 45 sec with 10% DMSO for 32cyles, using *Taq* polymerase.

Genotyping of double mutants was performed by Southern blot using 5'external probes. The same probe was used for detection of the *Sim1⁻* and *Sim1ᵗˡᶻ* alleles. This probe hybridizes to a 5.2-kb BamHI fragment of the wild-type *Sim1* allele, to a 3.4-kb BamHI fragment of the *Sim1⁻* allele and to a 4.5-kb fragment of the *Sim1ᵗˡᶻ* allele. The *Sim2* probe hybridizes to an 11-kb EcoRI fragment of the wild-type *Sim2* allele and to a 12-kb EcoRI fragment of the *Sim2⁻* allele.

C112k mice, which were derived at the Oak Ridge National Laboratory, carry a microdeletion encompassing *Arnt2* (Michaud et al., 2000). The anterior hypothalamus defect maps to a 320-350 kb region of which the *Arnt2*

structural genes spans 140-170 kb. Wild-type and heterozygote embryos were distinguished from homozygotes by the lack of eye pigmentation in the latter.

Histology, in situ hybridization, β-galactosidase staining and DiI labelling

All the analyses were done on at least two different embryos of the same stage and with the same genotype. For histology, embryo and newborn brains were fixed in Carnoy's fluid, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin. In situ hybridization was performed on paraffin sections, as previously described (Michaud et al., 1998). The following probes were generous gifts: *Foxb1* (P. Lebowski); *Nkx2.1* (J. Rubenstein); *Robo1*, *Robo2*, *Slit1*, *Slit2* and *Slit3* (M. Tessier-Lavigne); *Rig-1* (A. Chédotal); *Sim1* and *Sim2* (C-M Fan). The *Lhx1* probe was generated by RTPCR. Whole brains stained for β-galactosidase activity were sectioned at 100 μm with a vibratome. DiI crystals (Molecular Probes Inc.) were inserted in the MB of E14.5 brains fixed with 4% paraformaldehyde. These brains were incubated in paraformaldehyde for one week at room temperature and then sectioned at 100 μm with a vibratome.

Results

Development of mammillary body projections requires Sim1 and Sim2.
Extrapolation from birth dating studies performed in rats suggests that MB neurons are born between E10.5 and E13.5 in mice, exiting the cell cycle to migrate from the ventricular layer, which contains their progenitors, into the mantle layer in which they complete their differentiation (Altman and Bayer, 1978). Previous studies have shown that *Sim1* and *Sim2* start to be expressed in the neuroepithelium that will give rise to the MB at E9.5, before the birth of their first neurons (Fan et al., 1996). We performed a detailed comparison of their expression patterns in the prospective MB at latter stages because such description was lacking. At E10.5, *Sim1* is strongly expressed in the mantle

layer, which contains the first born MB neurons, and at weaker levels in the ventricular layer (Fig. 1A). At that stage, *Sim2* is co-expressed with *Sim1* in the ventricular layer whereas its expression in the mantle layer is much weaker (Fig. 1B). At E11.5 and E12.5, *Sim1* is mainly expressed in the whole mantle layer containing the prospective MB and more discretely in the adjacent ventricular zone. In contrast, *Sim2* transcripts are found in abundance in the ventricular zone and in the medial aspect of the mantle layer (Fig. 1C-F). At these stages, *Sim1* and *Sim2* are thus co-expressed in the ventricular layer and in the medial aspect of the mantle layer, which presumably contains neurons of latter generations. At E14.5, *Sim1* shows the same expression pattern whereas *Sim2* expression becomes weak and restricted to the ventricular layer (Fig. 1G,H). At E18.5, *Sim1* is expressed in the medial and lateral mammillary nuclei but we could not detect *Sim2* expression in the MB (not shown). Therefore, *Sim1* and *Sim2* are co-expressed in post-mitotic cells of the MB only during a short period, before E14.5, since *Sim2* expression in the mantle zone is transient whereas that of *Sim1* is continuous.

We next performed histological analysis of E18.5 embryos with different dosage of *Sim1* and *Sim2* in order to determine whether loss of these genes affects MB development. The MB appears histologically normal in all of these embryos, including in those with a lost of both *Sim1* and *Sim2*. Remarkably, the principal mammillary axonal tract (PMT), which gives rise to the MTEG and MTT, appears less prominent in $Sim1^{-/-};Sim2^{+/+}$ embryos compared to control or $Sim1^{+/+};Sim2^{-/-}$ embryos (Figs. 2,3A-F). However, *Sim2* is also required for the development of MB axons since *Sim1/Sim2* double mutants show a thin PMT and no detectable MTT, a more severe phenotype than that observed in $Sim1^{-/-};Sim2^{+/+}$ mice (Fig. 3I,L). $Sim1^{+/-};Sim2^{-/-}$ and $Sim1^{-/-};Sim2^{+/-}$ embryos have a MB phenotype comparable to that of $Sim1^{-/-};Sim2^{+/+}$ embryos (Fig. 3G,H,JK). All together, these results indicate that both *Sim1* and *Sim2* are required for MB axonal development, with *Sim1* having a predominant role over *Sim2*.

ARNT2, the bHLH-PAS dimerizing partner of SIM1 and presumably of SIM2 for anterior hypothalamus development, is expressed extensively in the CNS, including in the developing MB. We determined whether ARNT2 acts as a dimerizing partner of SIM1 and SIM2 for MB axonal development by comparing histologically the brains of E18.5 wild-type and *Cl12k* homozygous embryos, which carry a microdeletion encompassing the *Arnt2* locus (Michaud et al., 2000). Surprisingly, we found that the MTT and MTEG are intact in these *Cl12k* mutants. All together, these observations suggest that another dimerizing partner interacts with SIM1 and SIM2 for MB axonal development.

A Sim1^{tlacz} allele allows staining of mammillary body axons.

In order to further characterize the axonal projections originating from *Sim1* expressing cells, we generated a new targeted allele of *Sim1* (*Sim1^{tlz}*) in which the initiation codon, the basic and the HLH domains were replaced by a *Tau-lacZ* fusion gene (Fig. 4A). The targeted region overlaps with that of the initial *Sim1* mutant allele (*Sim1^-*), in which the initiation codon and the basic domain were deleted. This *Sim1^{tlz}* allele, predicted to be a null, would allow us to stain the MB axons that express *Sim1* and follow their fate in the context of a decrease of *Sim1* and/or *Sim2*. Using a double selection strategy, we obtained 12/140 (9 %) ES clones in which the *Sim1* locus underwent homologous recombination. One of these clones was used to generate a male chimera which was crossed to a C57Bl/6 female, resulting in germline transmission of the targeted allele (Fig. 4B). Mice homozygous for this allele show the same phenotypes as those described in mice with the previously described *Sim1^-* allele: *Sim1^{tlz/tlz}* mice die shortly after birth with a severe defect of the PVN/SON/APV (data not shown). Also, the pattern of lacZ staining in the brain of *Sim1^{tlz/+}* embryos and newborn mice was comparable to the distribution of *Sim1* transcript (compare Fig. 1G and 5F). Finally, histological analysis showed that the MB of newborn *Sim1^{tlz/tlz};Sim2^{-/-}* mice is preserved

whereas its MTT and PMT are not detectable (Fig. 4D,E). All together, these results indicate that the $Sim1^{tlz}$ allele is suitable to study the impact of $Sim1$ function during MB development.

Sim1/Sim2 mutant axons are directed towards the midline.

We next stained E14.5 brains with variable dosage of the $Sim1^{tlz}$ and $Sim2^-$ alleles for β-galactosidase activity. The PMTs of $Sim1^{tlz/+};Sim2^{+/-}$ and $Sim1^{tlz/+};Sim2^{-/-}$ brains are clearly recognizable and their sizes are comparable (Fig. 5A,B,F,G,K,L,P,Q). At the level of the anterior MB, the PMT bundle progresses in a domain dorsal to the MB that produces the LacZ activity. Anteriorly, only one bundle of MB axons appears to develop whereas more posteriorly, several bundles merge to form the main PMT bundle before it leaves the MB domain (compare Fig. 5A,B and P,Q). In contrast, the PMT is greatly reduced in $Sim1^{tlz/tlz};Sim2^{+/+}$ brains (Fig. 5C,H,M,R). At the most posterior aspect of the MB, a bundle presumably corresponding to the PMT is recognizable but is accompanied by additional axons, originating more laterally, which project towards the midline and become splayed on their way to reach it. These findings are more striking in $Sim1^{tlz/tlz};Sim2^{+/-}$ and $Sim1^{tlz/tlz};Sim2^{-/-}$ brains in which a PMT is barely recognizable and a group of axons similar to that observed in $Sim1^{tlz/tlz};Sim2^{+/+}$ brains but more prominent is found (Fig. 5D,E,I,J,N,O,S,T). At the most posterior level of the MB, these axons cross the midline ventrocaudally to the third ventricle. In embryos lacking two copies of $Sim1$, the number of bundles arising in the MB appears somewhat decreased. Of note, we did not observe consistent changes of tau-lacz expression according to the different genotypes in the region located dorsally to the MB, in which PMT axons progress.

No difference between the pattern of lacZ staining of axonal projections found in genetic compounds for the $Sim1^-$ and $Sim1^{tlz}$ alleles versus those of homozygotes was detected, consistent with the assumption that the tau-lacz fusion gene does not generally affect axon development in mice (data not

shown). In order to further validate the use of the $Sim1^{tlz}$ allele to label MB axons, we inserted crystals of DiI into the MB of E14.5 $Sim1^{+/-};Sim2^{+/-}$ and $Sim1^{-/-};Sim2^{-/-}$ embryos and compared their pattern of axonal projections. The PMT was clearly labeled in control embryos and very rare axons could be seen progressing towards the midline (Fig. 6 A,B). In contrast, most MB axons are directed towards the midline in $Sim1^{-/-};Sim2^{-/-}$ embryos. The axons became splayed as they reached the midline but a subset of these form a bundle crossing the midline ventrally (Fig. 6 C,D). This pattern is similar to that observed with the $Sim1^{tlz}$ allele, indicating that the latter is expressed in most MB axons and again supporting our conclusion that its expression *per se* does not affect MB axons development.

We next used this combination of $Sim1^-$ and $Sim1^{tlz}$ alleles to study MB axonal projections in E11.5 embryos, shortly after they appeared. In $Sim1^{tlz/+};Sim2^{+/-}$ and $Sim1^{tlz/+};Sim2^{-/-}$ embryos, MB axons form bundles converging dorsally within a tau-lacz expression domain (Fig. 7 A,B). These bundles are less prominent but are nevertheless oriented dorsally in $Sim1^{tlz/-};Sim2^{+/-}$ embryo (Fig. 7 C). In contrast, MB axons are not detectable in $Sim1^{tlz/-};Sim2^{-/-}$ embryos, suggesting either that their development is delayed or that the axons are splayed, not forming bundles and thus escaping detection by the β-galactosidase staining (Fig. 7 D). The loss of $Sim1$ and $Sim2$ therefore affects the early development of MB axons.

Sim1/Sim2 mutant neurons are generated and survive until E18.5.

The MB appears histologically intact in *Sim1/Sim2* double mutants. In order to determine whether *Sim1/Sim2* affects the differentiation of the MB, we performed marker analysis. The *Sim1* mutant allele is a null but does not interfere with the production and the stability of its transcript, which can be used to follow the fate of *Sim1* mutant cells (Michaud et al., 1998). We found that the expression of the *Sim1* mutant transcript in the MB of E12.5 $Sim1^{-/-};Sim2^{-/-}$ embryos is comparable to that of controls, consistent with the fact that

the production of the Tau-lacZ fusion protein is maintained in the MB of E14.5 mutant embryos (Fig. 8 A,B). Similarly, we found that *Lhx1* and *Nkx2.1* expression is maintained in the MB of E12.5 double mutants (Fig. 8 C-F). Of note, the expression of *Sim1*, *Lhx1* and *Nkx2.1* is also maintained in a domain dorsal to the E12.5 MB in which the PMT progresses. Since *Sim1*, *Lhx1* and *Nkx2.1* are expressed in virtually all MB cells, the loss of *Sim1* and *Sim2* thus does not seem to affect the generation and survival of postmitotic neurons in the developing MB. In contrast, *Foxb1* expression is dramatically decreased in the prospective MB and in the dorsal domain of $Sim1^{-/-};Sim2^{-/-}$ embryos, but not in those of embryos with at least one allele of *Sim1/Sim2*, indicating that *Sim1/Sim2* acts upstream of *Foxb1* (Fig. 8 G,H and 9 E-H). At E18.5, *Lhx1* expression remains robust in the MB of double mutants whereas the expression of *Sim1* mutant transcript is slightly decreased (Fig. 9C-D).

The conservation of *Sim1* expression in the MB of double mutants indicates that *Sim1* expression does not require the presence of *Sim2*. Conversely, we found that *Sim2* expression is maintained in the MB of E12.5 $Sim1^{-/-}$ embryos (data not shown). Therefore, *Sim1* and *Sim2* function along compensatory but not hierarchical pathways during MB development.

Sim1 and Sim2 repress Rig1/Robo3 expression in the developing mammillary body.

SLIT2, a secreted molecule that induces repulsion of axons that express its receptor ROBO1 or ROBO2, is produced in the ventricular layer of the hypothalamus. Dopaminergic axons, which normally progress longitudinally, are attracted by the ventral midline of the hypothalamus in *Slit2* mutant embryos (Bagri et al., 2002). A similar phenotype is observed in *Nkx2.1* mutant embryos, one study showing that dopaminergic axons actually cross the midline in the ventral part of the caudal hypothalamus (Marin et al., 2002; Kawano et al., 2003). *Slit2* expression in the ventral hypothalamus is downregulated in these mutants, suggesting that the decrease of this factor

causes the abnormal axonal targeting. Interestingly, *Slit* expression in the CNS midline cells of the fly embryo is directly controlled by *sim*, a homolog of *Sim1* and *Sim2* (Wharton, Jr. and Crews, 1993). In order to explore the possibility that such an interaction has been conserved in vertebrates, we examined the expression of *Slit1, Slit2, Slit3* at E11.5 as well as at E12.5, when mutant MB axons start to grow towards the midline. We found that *Slit1* is expressed in a patch of the ventricular layer dorsal to the prospective MB corresponding to the area of the midline towards which mutant axons grow whereas *Slit2* is expressed in the ventricular layer adjacent to and dorsal to the MB (Fig. 10 A,C,E,G and data not shown). *Slit1* and *Slit2* expression in the caudal hypothalamus was not modified in E11.5 and E12.5 *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos (Fig 10 B,D,F,H and data not shown). *Slit3* was not expressed in this region in *Sim1$^{+/+}$;Sim2$^{+/+}$* or *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos (data not shown). These observations indicate that *Sim1* and *Sim2* do not regulate the expression of the *Slit* genes in the developing MB.

In order to determine whether other components of this molecular system are involved in the genesis of the phenotype, we next compared the expression of *Robo1, Robo2* and *Rig1/Robo3* in the MB of E11.5 and E12.5 wild-type and *Sim1/Sim2* mutant embryos. *Robo1* is expressed almost ubiquitously in the caudal hypothalamus, with stronger levels found in the prospective MB (Fig. 10 I,K and data not shown). *Robo2* is not expressed in the MB but it is in more dorsal regions (not shown). The expression of *Robo1* and *Robo2* in the caudal hypothalamus was not changed in E11.5 and E12.5 *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos (Fig. 10 J,L and data not shown). *Rig-1/Robo3* is a distant homolog of *Robo1* and *Robo2* that appears to function cell autonomously to inhibit *Slit* signalling by a mechanism that has not yet been resolved (Sabatier et al., 2004; Marillat et al. 2004). At E11.5, *Rig-1* is expressed in a small patch that is contained within the anterior aspect of the *Sim1* domain but its expression is not found more posteriorly (Fig. 10 U,W,Y,A'). In *Sim1$^{-/-}$;Sim2$^{-/-}$* as well as in *Sim1$^{-/-}$;Sim2$^{+/-}$* embryos, this anterior domain of expression is dramatically expanded

whereas *Rig-1* expression becomes detectable in the posterior MB (Fig. 10 V,X,Z,B' and data not shown). At E12.5, *Rig-1* is expressed in a narrow domain that extends obliquely within the mantle layer of the anterior MB (Fig. 10 M). Its medial half overlaps with the dorsal aspect of the MB prospective domain, as indicated by comparison with the *Sim1* expression pattern, whereas its lateral half is located more dorsally. In *Sim1$^{-/-}$;Sim2$^{-/-}$* as well as in *Sim1$^{-/-}$;Sim2$^{+/-}$* embryos, *Rig-1* is expressed ectopically in the ventrolateral aspect of the anterior MB domain whereas its expression dorsally to this domain is reduced (Fig. 10 N and data not shown). At the level of the posterior MB, *Rig-1* expression is barely detectable in control littermates (Fig. 10 O). In contrast, *Rig-1* expression is clearly detectable in the posterior MB of *Sim1$^{-/-}$;Sim2$^{-/-}$* and *Sim1$^{-/-}$;Sim2$^{+/-}$* embryos, being restricted to its lateral aspect (Fig. 10 P). In summary, *Rig-1* is expressed ectopically in the developing MB of *Sim1/Sim2* double mutants raising the possibility that it contributes to the axonal defects by decreasing the sensitivity of MB axons to *Slit* signalling.

Discussion

The loss of *Sim1/Sim2* produces a complex axonal phenotype that is characterized by hypodevelopment of the PMT and the presence of axons directed towards the midline or crossing it. The severity of these two features increases progressively with the greater dosage of *Sim1/Sim2* mutant alleles, the size of the PMT diminishing and the number of abnormally-oriented axons increasing concomitantly. SIM1 and SIM2 are thus novel examples of a growing group of transcription factors that act as critical regulators of axonal morphogenesis and connectivity and represent critical reference points for further dissection of axon development in the MB. Interestingly, the optic lobes of *sim* mutant flies show aberrant axonal projections, while the neurons in which it is expressed are present, raising the possibility that *Sim1* function

for axonal development has been conserved through evolution (Pielage et al., 2002).

Requirement of Sim1 and Sim2 for MB axonal development

Axonal growth cones are submitted to the influence of attractive and repulsive signals that guide their progression in the developing brain (reviewed in (Giger and Kolodkin, 2001)). Recent studies indicate that several groups of longitudinal axons are repulsed from the midline by SLIT during development (Bagri et al., 2002). *Slit1* and *Slit2* are expressed in the ventricular layer adjacent to the MB as well as dorsally whereas *Robo1* is expressed in the MB. These observations suggest that *Slit* signalling might also repulse MB axonal cones from the midline, a possibility that needs to be validated through gain- or loss-of function experiments in mice. Interestingly, we have found that *Rig-1/Robo3* expression is upregulated in the prospective MB domain of *Sim1$^{-/-}$;Sim2$^{+/-}$* and *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos. *Rig-1* functions cell autonomously to inhibit *Slit* signalling in neurons of the spinal cord and of the hindbrain (Sabatier et al., 2004; Marillat et al., 2004). We propose that loss of repression of *Rig-1* in *Sim1/Sim2* mutant MB neurons decrease the responsiveness of their axons to SLIT, resulting in their growth towards the midline. Consistent with this possibility, E14.5 *Sim1/Sim2* mutant axons originate form the lateral and posterior MB where ectopic *Rig-1* expression is detected at E12.5.

Ectopic expression of *Rig-1* in the developing MB, however, does not readily explain other aspects of the axonal phenotype of *Sim1/Sim2* embryos. At E11.5, mutant axons do not form clearly recognizable bundles, suggesting a decrease of MB projections, while there are not yet axons directed towards the midline. At E14.5, bundles projecting towards the midline are present in the posterior MB of mutant embryos but there are no visible axons originating from the anterior MB of these mutants. These observations suggest a decrease of axonal growth in *Sim1/Sim2* mutants that cannot be simply explained by an abnormal interaction with the midline. One possibility would be that SLIT2,

produced by the ventricular layer that lies ventromedially, repulses MB axons dorsally contributing to their polarized growth. Of note, whereas *Rig-1* expression is upregulated in the MB domain of mutant embryos, we found that it is decreased in a domain located immediately dorsal to that of the MB. The decrease of *Rig-1* expression in this dorsal domain could reflect distinct regulatory interactions or impaired development of these cells.

Other explanations can be proposed to account for the decrease of axonal growth in the double mutants. For instance, *Sim1/Sim2* could function cell autonomously to regulate the expression of signalling components required for response to an attracting signal. Alternatively, *Sim1* and *Sim2* would be required in the environment in which the MB axons progress to control the expression of an attractive or of a permissive signal. Indeed, *Sim1* is expressed in a domain dorsal to the MB, which contains the PMT from the time of its appearance. Before E11.5, *Sim1* and *Sim2* expression overlaps in a region of the ventricular layer that presumably gives rise to MB and the dorsal domain. At later stages, they are co-expressed in the lateral ventricular layer and in the medial aspect of the mantle layer of the prospective MB but do not overlap in the dorsal domain. If *Sim1* and *Sim2* are indeed required in the dorsal domain for correct development of MB axons, one would have to postulate that they function at an early stage in precursors of cells of the dorsal domain. The fact that *Foxb1* expression in this domain is downregulated in the *Sim1/Sim2* double mutant, but not in *Sim1$^{-/-}$;Sim2$^{+/+}$* embryos, indicate that *Sim2* can influence expression in these dorsal cells. Finally, *Sim1* could function in both the axons and in their surrounding tissues, as it was shown for the transcription factor lola in the developing fly (Crowner et al., 2002).

Cascade of transcription factors controlling MB development
Signals produced by axial mesodermal structures, such as *Shh* and *Bmp7*, are required to induce *Nkx2.1* expression in the neuroepithelium that will give rise to ventral regions of the developing hypothalamus, including the MB (Kimura

et al., 1996; Ericson et al., 1995; Pabst et al., 2000). The MB and several ventromedial nuclei of the caudal hypothalamus do no develop in embryos with a loss of *Nkx2.1,* suggesting that it is required to specify the whole ventro-caudal hypothalamic anlage (Kimura et al., 1996; Marin et al., 2002). The fact that the MB domain of *Sim1* expression is dramatically reduced in *Nkx2.1* mutant embryos as early as E11.5 indicates that *Nkx2.1* functions upstream of *Sim1* for MB development (Marin et al., 2002). Consistent with this conclusion are our observations that *Sim1* and *Sim2* are not required for the generation and initial differentiation of MB neurons and that *Nkx2.1* expression is not affected by the loss of both *Sim1* and *Sim2*. Similarly, *Nkx2.2*, a close homolog of *Nkx2.1*, is required for *Sim1* expression in the developing ventral spinal cord, whereas *Nkx2.2* expression in this region is not affected by the loss of *Sim1* (Briscoe et al., 1999; Briscoe et al., 2000). Collectively, these observations suggest the existence of homologous pathways in these two ventral regions of the CNS along which the *Nkx2* and *Sim* genes would act.

We have found that *Foxb1* expression is greatly reduced in the MB of *Sim1/Sim2* double mutants. This observation raises the possibility that *Foxb1* mediates the effect of a decrease of *Sim1/Sim2* on MB axonal guidance. However, *Foxb1* mutant analysis does not support this possibility, since the loss of *Foxb1* function only affects MTT development (Alvarez-Bolado et al., 2000). Consistently, chimera analyses suggest that *Foxb1* is required in the dorsal thalamus for MTT formation. Moreover, we did not observe decrease of *Foxb1* expression in embryos with at least one allele of *Sim1/Sim2* despite the fact the axonal guidance abnormalities are observed in these embryos. The loss of *Foxb1*, however, might suggest that *Sim1* and *Sim2* are required to control aspects of MB differentiation other than axonal growth that were not revealed by our analysis.

Respective functions of bHLH-PAS proteins during MB development

The basic, HLH and PAS domains of SIM1 and SIM2 share high identity whereas their carboxy-terminal domains are poorly conserved. Consistent with the low identity of their carboxy-termini, SIM1 and SIM2 display distinct transcriptional properties in cultured cell system. The SIM1-ARNT/2 heterodimer transactivates reporter constructs via the ARNT carboxy-terminus (Moffett and Pelletier, 2000; Woods and Whitelaw, 2002). SIM1 does not have neither activation nor repression activity in this context. In contrast, SIM2-ARNT/2 does not activate transcription unless the carboxy-terminus of SIM2 is deleted. The carboxy-terminus of SIM2 appears to have a repressive function, which quenches the transactivating of ARNT (Moffett and Pelletier, 2000; Woods and Whitelaw, 2002). Since SIM1 and SIM2 compete for binding to ARNT/2 and to the DNA binding site, these different properties of SIM1 and SIM2 result in some transcriptional antagonism at least *in vitro* (Moffett and Pelletier, 2000).

Our study indicates, however, that *Sim1* and *Sim2* can compensate for the absence of each other, the former playing a predominant role over the latter during MB development. We did not observe reduction of *Sim2* expression in the MB of *Sim1*$^{-/-}$ embryos and vice-versa, suggesting that the interaction between *Sim1* and *Sim2* is not hierarchical. All together, these results indicate that *Sim1* and *Sim2* can play similar role in vivo, even though their C-termini have diverged considerably. There are other lines of evidence supporting this conclusion. Overexpression of *Sim1* or *Sim2* using a *Wnt1* enhancer activates *Shh* expression in the mouse midbrain, demonstrating that *Sim1* and *Sim2* can act similarly in a given embryonic context (Epstein et al., 2000). Moreover, *Sim1* can compensate for the absence of *Sim2*, albeit ineffectively, during differentiation of the PVN. The interplay between *Sim1* and *Sim2* is, however, complex in the developing PVN since mutant analysis indicates that they also control different aspects of PVN neuronal differentiation and that *Sim1* is required for *Sim2* expression (Goshu et al., 2004). Recent studies provide other examples of interaction between bHLH-PAS proteins during

development. For instance, *Dysfusion* downregulates *trachealess* expression in the developing trachea of the fly and *Nxf* compete with *Sim2* for binding to elements that regulate the expression of a gene engaged in dendritic-cytoskeleton modulation at synapses (Jiang and Crews, 2003; Ooe et al., 2004). It will be interesting to determine whether these or other bHLH-PAS interact with *Sim1/Sim2* during the development of the MB.

Biochemical, expression and mutant studies indicate that ARNT2 is required for PVN development by acting as the dimerizing partner of SIM1. It appears likely that SIM2 also heterodimerizes with ARNT2 in the PVN, since they can physically interact (Goshu et al., 2004). However, because the PVN phenotype of *Arnt2*$^{-/-}$ mice is identical to that of *Sim1*$^{-/-}$ mice and more severe than that of *Sim2*$^{-/-}$ mice, it has not been formally shown that SIM2 controls PVN neuronal differentiation through this interaction. Surprisingly, our analysis indicates that MB axonal tracts can develop in the absence of *Arnt2*. A homologue, *Arnt,* could compensate for the absence of *Arnt2* but its expression level is particularly low in the MB of wild-type and *Arnt2* mutant embryos (Caqueret et al. unpublished observations). Alternatively, SIM1 and SIM2 could dimerize with a member of another subgroup of partners such as BMAL1 or BMAL2, raising the possibility that the use of different partners could influence the function of SIM1/SIM2. Such heterogeneity in the composition of the SIM1 and SIM2 complexes could account for the discrepancy between their respective *in vivo* and *in vitro* transcriptional activities.

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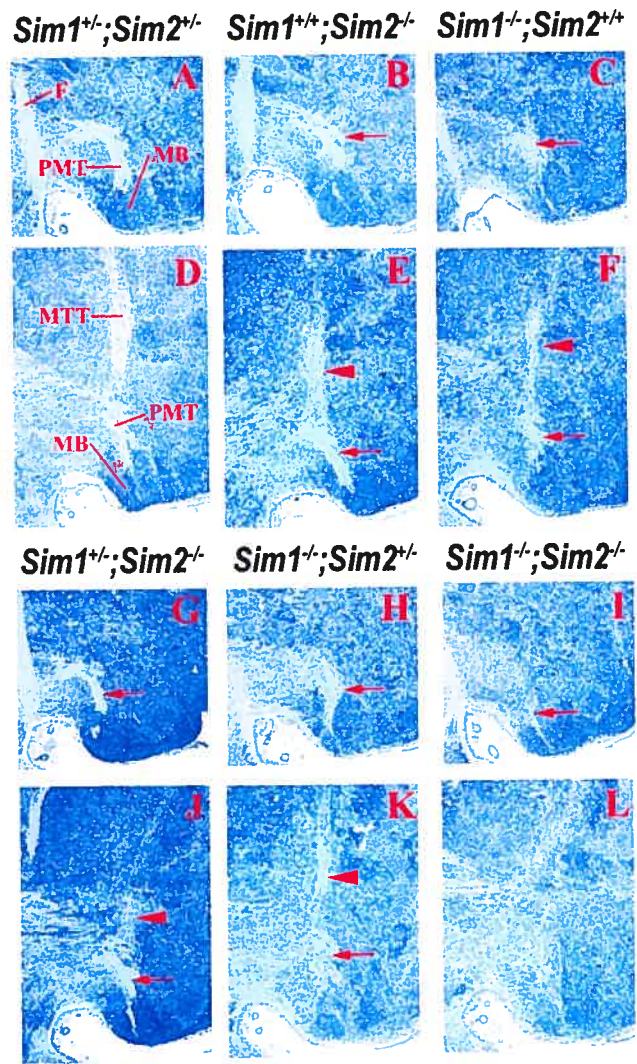
Sim1 Sim2
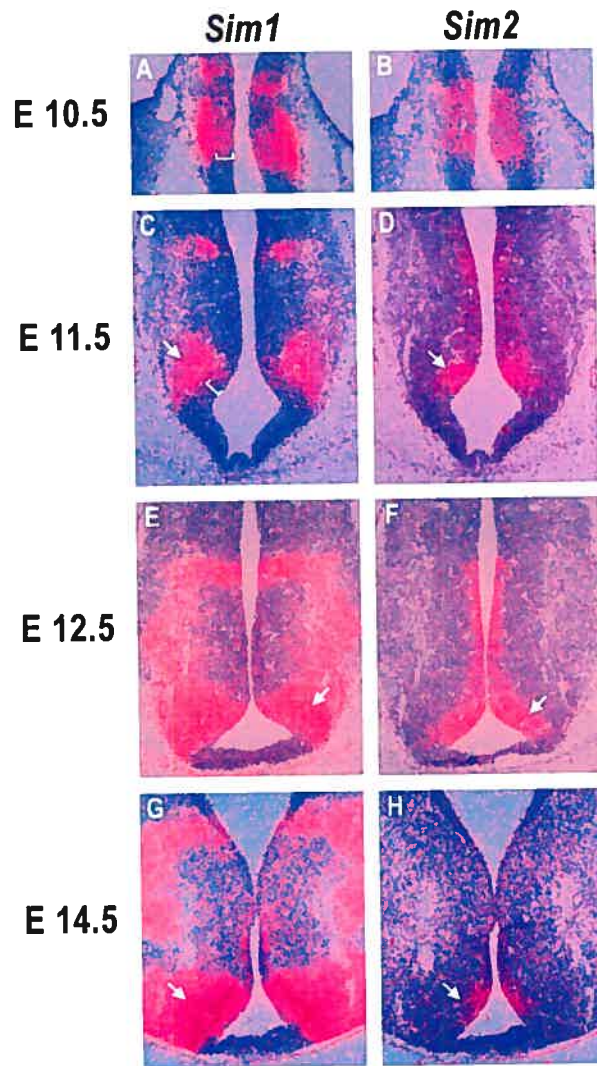
E 10.5

E 11.5

E 12.5

E 14.5

Figure 1.

Figure 1. *Coexpression of Sim1 and Sim2 in the developing mammillary body.* Adjacent coronal sections through the prospective MB of E10.5 (**A,B**), E11.5 (**C,D**), E12.5 (**E,F**) and E14.5 (**G,H**) wild-type embryos were hybridized either with *Sim1* (**A,C,E,G**) or *Sim2* (**B,D,F,H**). (**A,B**) At 10.5, *Sim1* is expressed in the lateral aspect of the neuroepithelium, which presumably corresponds to the mantle layer, but less strongly in the medial aspect (bracket), which corresponds to the ventricular layer. *Sim2* is mainly expressed in this medial domain. (**C-F**) At E11.5 and E12.5, *Sim1* is expressed strongly in the mantle layer, this domain corresponding to the prospective MB, but also weakly in the ventricular layer (bracket). Sim2 is expressed in the ventricular layer and in the medial aspect of the mantle layer of the prospective MB. (**G,H**) At E14.5, *Sim1* shows the same expression pattern. *Sim2* expression has decreased in intensity and becomes restricted to the ventricular layer. The arrows indicate the prospective MB.
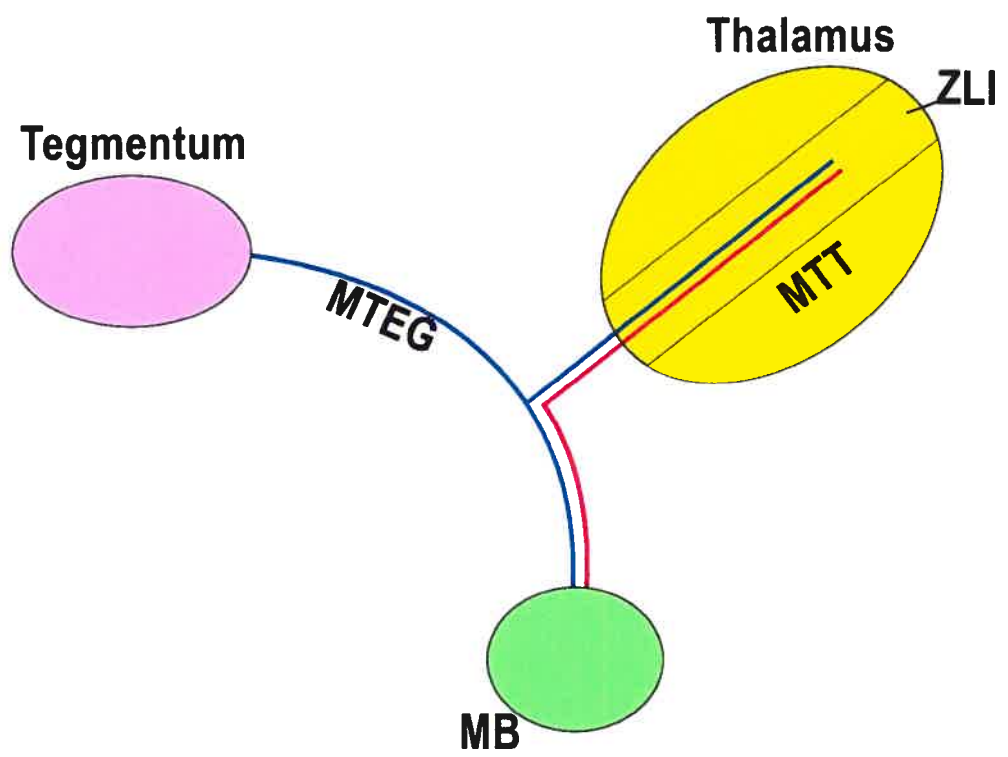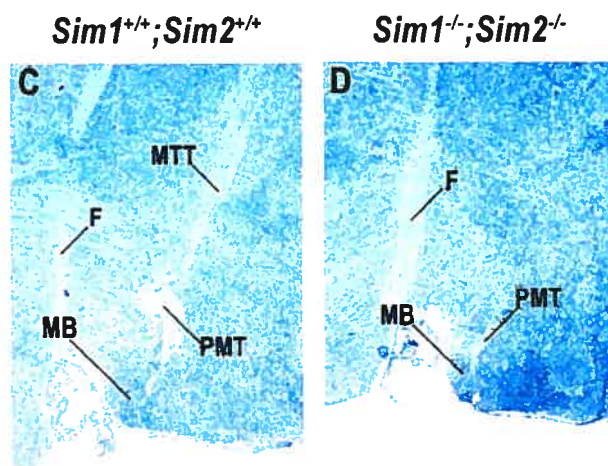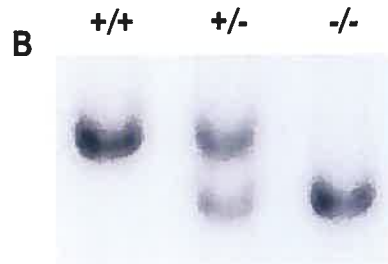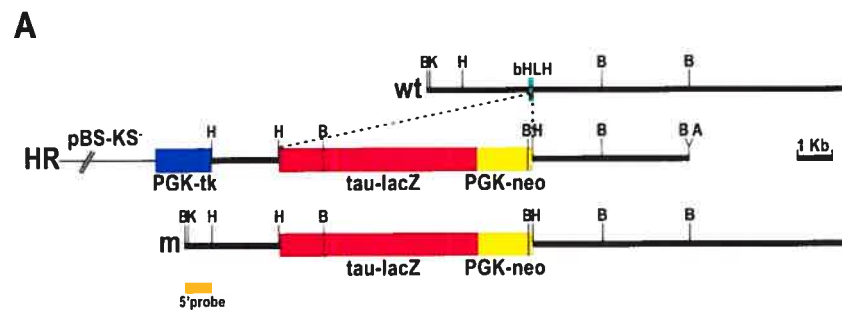
Figure 2.

Figure 2. *Organization of the mammillary body projections.* The left side of the brain is represented in a sagittal perspective. Rostral is to the right. The principal mammillary tract (PMT) gives rise to the mammillotegmental (MTEG) and mammillothalamic tract (MTT).
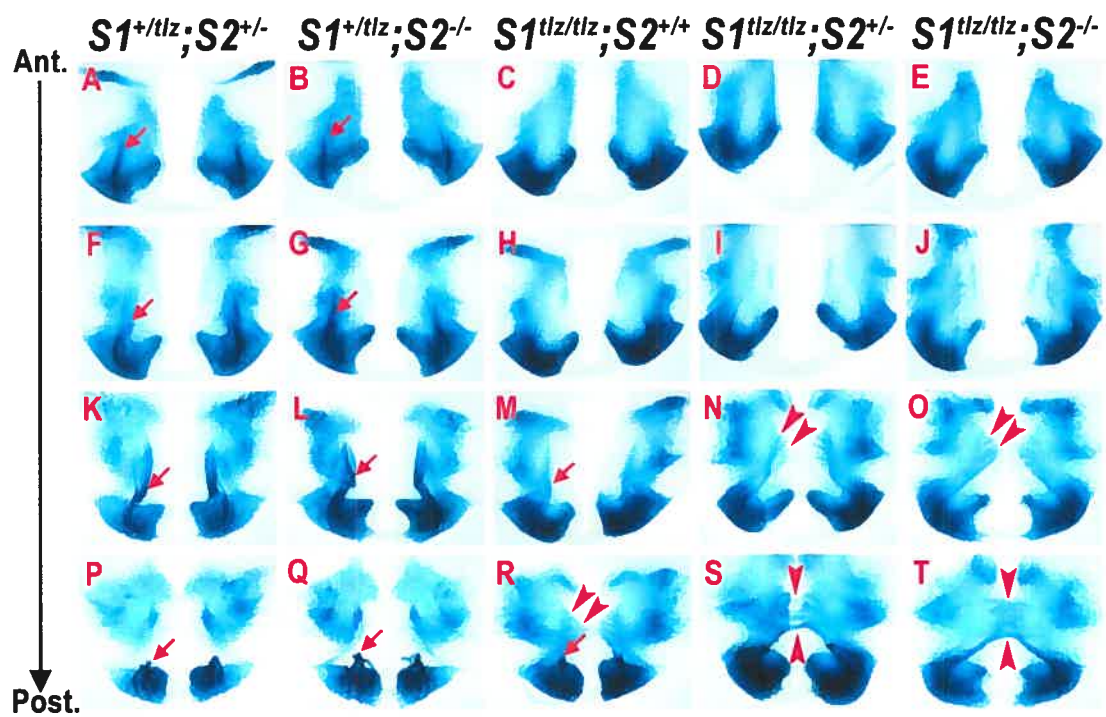
Figure 3.

Figure 3. *MTEG and MTT development affected by Sim1/Sim2 gene dosage.*
E18.5 brains with various genotypes were sectioned sagittally and stained with
hematoxylin. The upper panels (**A,B,C,G,H,I**) represent medial sections
containing the PMT whereas the lower panels correspond to lateral sections
that include the MTT and the PMT. The MTEG is not readily detectable on
sagittal sections because of its orientation. The PMT is indicated by arrows
whereas the MTT by arrowheads. The PMT and MTT are well developed in
Sim1$^{+/-}$;Sim2$^{+/-}$ (**A,D**) and *Sim1$^{+/+}$;Sim2$^{-/-}$* (**B,E**) embryos whereas they were
thinner in *Sim1$^{-/-}$;Sim2$^{+/+}$* (**C,F**), *Sim1$^{+/-}$;Sim2$^{-/-}$* (**G,J**) and *Sim1$^{-/-}$;Sim2$^{+/-}$*
(**H,K**) embryos. The PMT and MTT were barely detectable in *Sim1$^{-/-}$;Sim2$^{-/-}$*
embryos (**I,L**). In all cases, the MB appeared histologically present.

A

wt — BK H bHLH B B

HR — pBS-KS⁻ H H B B H B A 1 Kb
PGK-tk — tau-lacZ — PGK-neo

m — BK H H B B H B B
tau-lacZ — PGK-neo
5'probe

B +/+ +/- -/-

Sim1⁺/⁺;Sim2⁺/⁺   *Sim1⁻/⁻;Sim2⁻/⁻*

C MTT F MB PMT

D F MB PMT

Figure 4.

Figure 4. *Creation of a Sim1 allele expressing Tau-lacZ.* (**A**) Schematic representation of the *Sim1* locus (wt), of the targeting vector (HR), and of the *Sim1* mutant allele (m). Homologous recombination replaces the initiation codon, the basic and the HLH domains by a Tau-lacZ fusion gene. The 5' external probe is indicated. Restriction enzymes: (B) *BamHI*, (H) *HindIII*. (**B**) Southern blot analysis of genomic DNA from $Sim1^{+/+}$, $Sim1^{tlz/+}$ and $Sim1^{tlz/tlz}$ mice. The 5' probe detects a wild-type 5.2-kb *BamHI* fragment and a mutant 4.5-kb *BamHI* fragment. (**C,D**) Sagittal sections through the MB of $Sim1^{+/+};Sim2^{+/+}$ and $Sim1^{-/-};Sim2^{-/-}$ E18.5 embryos which have been stained with hematoxylin. Loss of the $Sim1^{tlz}$ allele interferes with the development of MB axons as did the $Sim1^{-}$ allele.
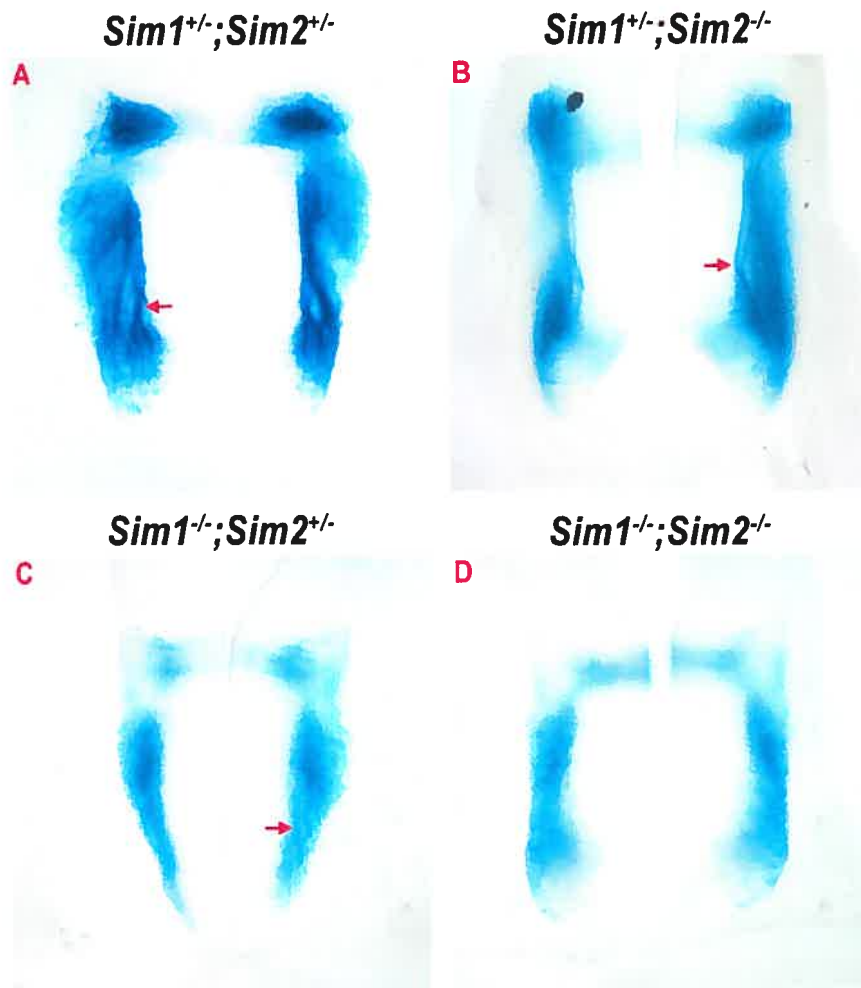
Figure 5.

Figure 5. *LacZ staining of mammillary body axonal projections in E14.5 Sim1/Sim2 mutant embryos.* E14.5 brains with various genotypes were stained for β-galactosidase activity and sectioned coronally. For each brain, four consecutive sections are shown, the most anterior being at the top of the figure. PMTs are indicated by the arrows whereas the abnormally targeted are indicated by the arrowheads. The loss of *Sim1* function is associated with a decrease of the PMT and the emergence of MB axons directed towards the midline. *Sim2* also contributes to this phenotype since the axonal abnormalities are more severe in $Sim1^{llz/llz}; Sim2^{-/-}$ than in $Sim1^{llz/llz}; Sim2^{+/+}$.

Sim1⁺ᐟ⁻;Sim2⁺ᐟ⁻

Sim1⁻ᐟ⁻;Sim2⁻ᐟ⁻
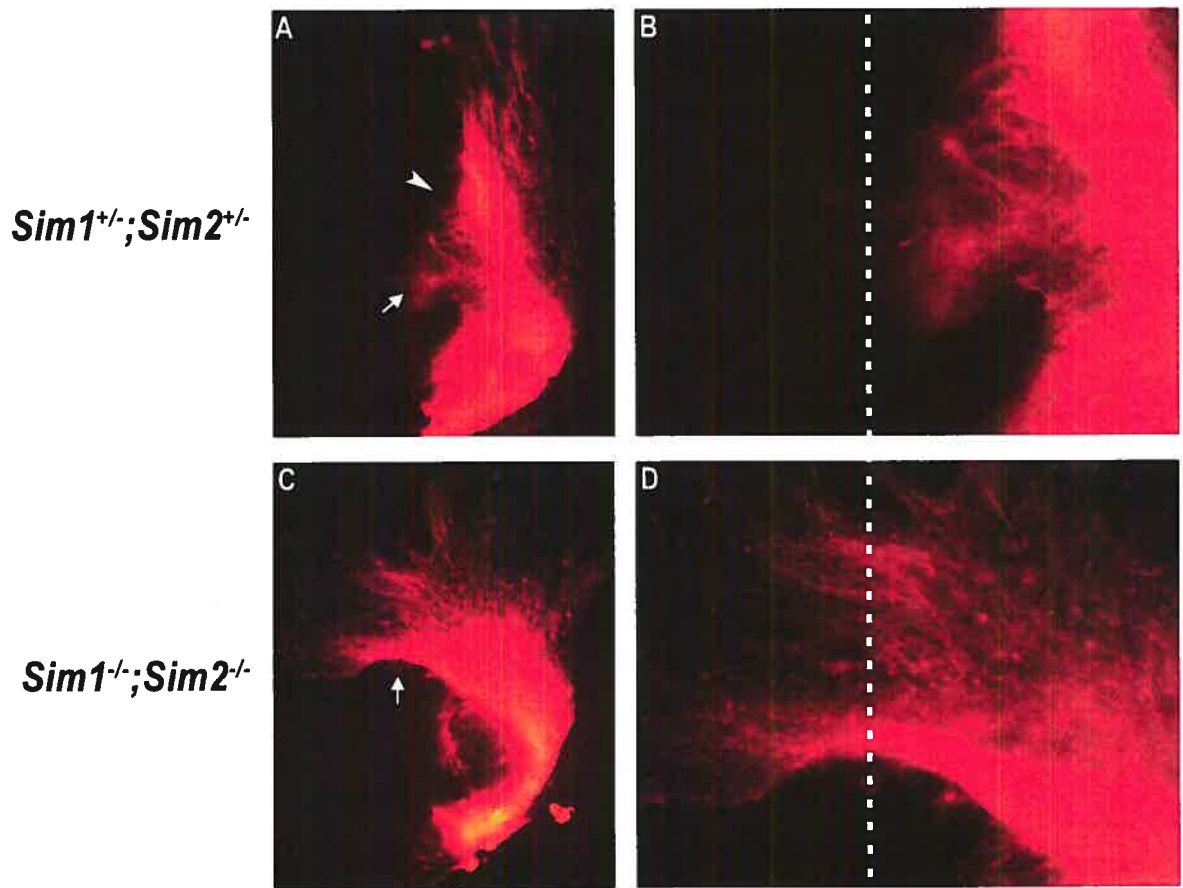
Figure 6.

Figure 6. *Abnormal targeting of mammillary body axons as revealed by DiI labelling.* Crystals of DiI were inserted in E14.5 brains of $Sim1^{+/-};Sim2^{+/-}$ (**A,B**) and $Sim1^{-/-};Sim2^{-/-}$ (**C,D**) embryos and sectioned after an incubation period of 2 weeks. (**B**) and (**D**) represent higher magnification of (**A**) and (**C**), respectively. The white line in (**B,D**) corresponds to the midline. The PMT (arrowhead) is recognizable in $Sim1^{+/-};Sim2^{+/-}$ but not in the double mutants. A few axons appear to progress towards the midline in $Sim1^{+/-};Sim2^{+/-}$ embryos (arrow) whereas a majority of them do so in $Sim1^{-/-};Sim2^{-/-}$ embryos.

Sim1⁺ᐟ⁻;Sim2⁺ᐟ⁻

Sim1⁺ᐟ⁻;Sim2⁻ᐟ⁻

Sim1⁻ᐟ⁻;Sim2⁺ᐟ⁻

Sim1⁻ᐟ⁻;Sim2⁻ᐟ⁻

Figure 7.

Figure 7. *LacZ staining of mammillary body axonal projections in E11.5 Sim1/Sim2 mutant embryos.* E11.5 brains with various genotypes, as indicated, were stained for β-galactosidase activity and sectioned coronally. Axonal bundles (arrows) are easily recognizable in $Sim1^{+/-};Sim2^{+/-}$ (**A**) and in $Sim1^{+/-};Sim2^{-/-}$ (**B**) embryos but decreased in $Sim1^{-/-};Sim2^{+/-}$ (**C**). No bundle was detected in $Sim1^{-/-};Sim2^{-/-}$ embryos (**D**). Note that axons progress in a domain that is stained. LacZ staining in the dorsal domain is weaker in C and D than in A and B because these thick sections of early embryos are from a slightly different plan.
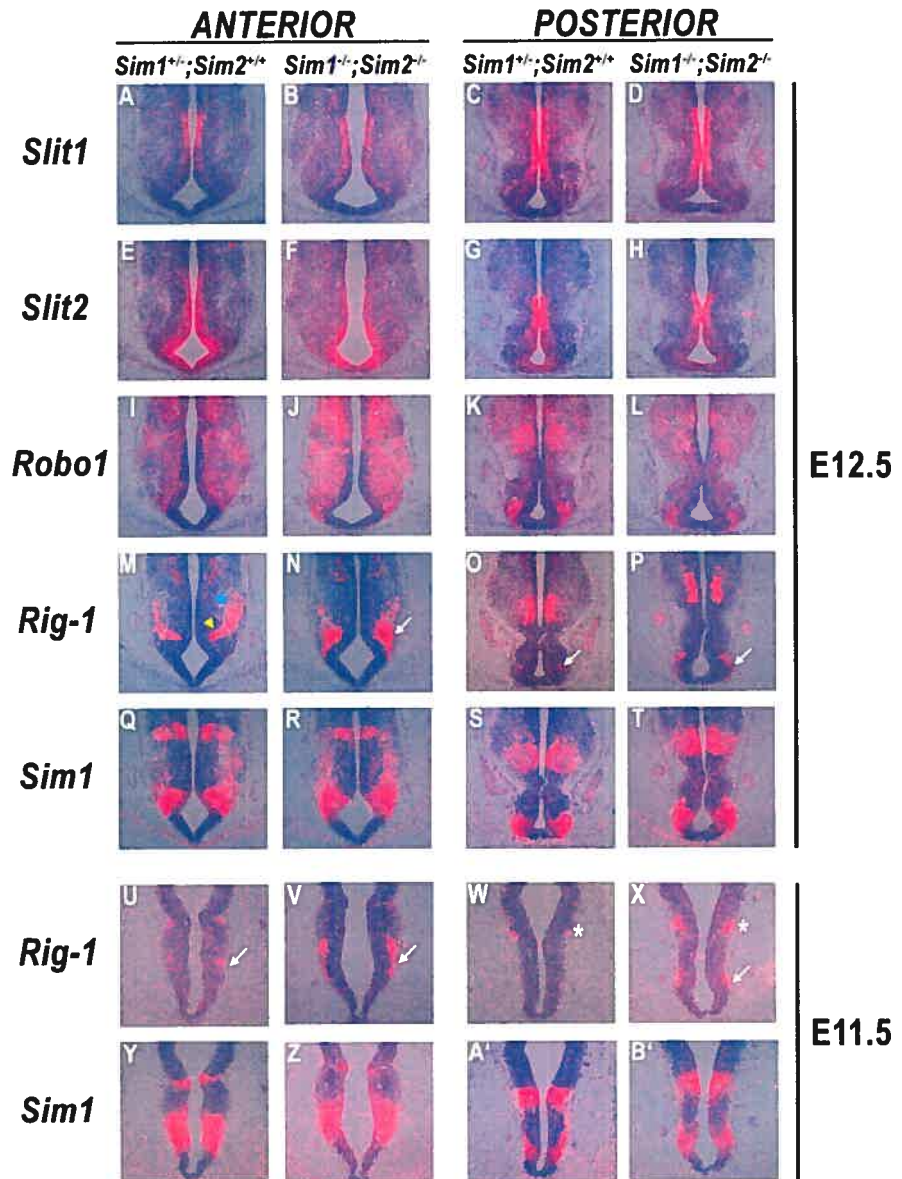
$Sim1^{+/+};Sim2^{+/+}$ $Sim1^{-/-};Sim2^{-/-}$

Sim1
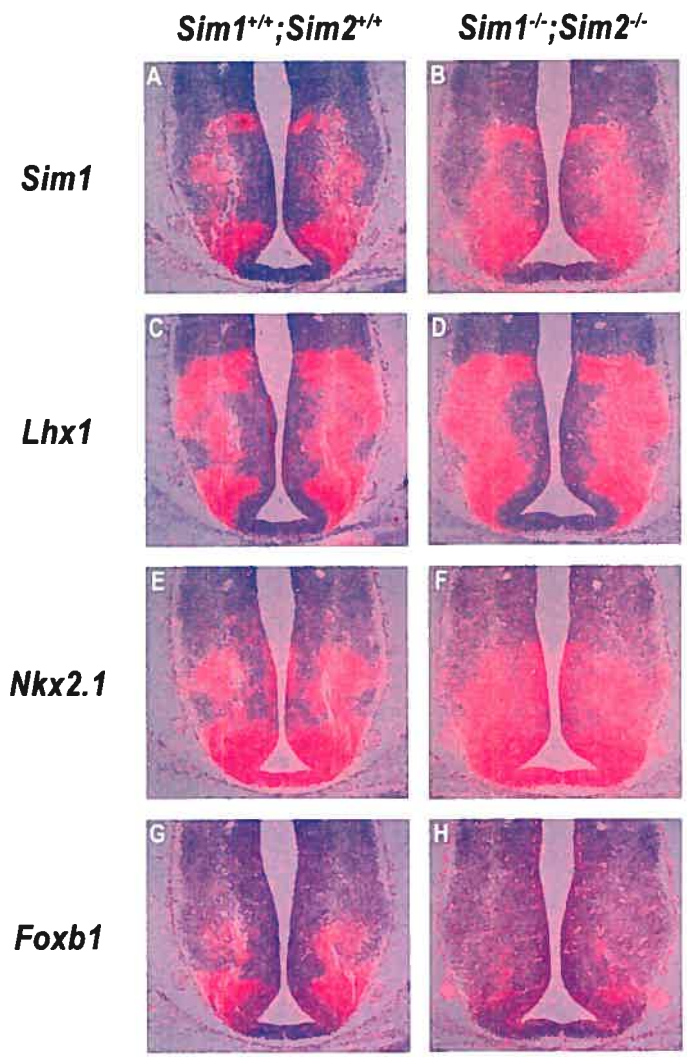
Lhx1

Nkx2.1

Foxb1

Figure 8.

Figure 8. *Loss of Foxb1 expression in E12.5 Sim1/Sim2 double mutants.* Coronal sections through the MB of E12.5 *Sim1*$^{+/+}$*;Sim2*$^{+/+}$ (**A,C,E,G**) and *Sim1*$^{-/-}$*;Sim2*$^{-/-}$ (**B,D,F,H**) embryos were hybridized either with a *Sim1* (**A,B**), *Lhx*1 (**C,D**), *Nkx2.1* (**E,F**) or a *Foxb1* (**G,H**) probe. The maintenance of *Sim1*, *Lhx1* and *Nkx2.1* expression in double mutants indicates that the loss of *Sim1/Sim2* does not affect early differentiation of MB neurons. *Sim1/Sim2* are, however, required to maintain *Foxb1* expression in the MB.
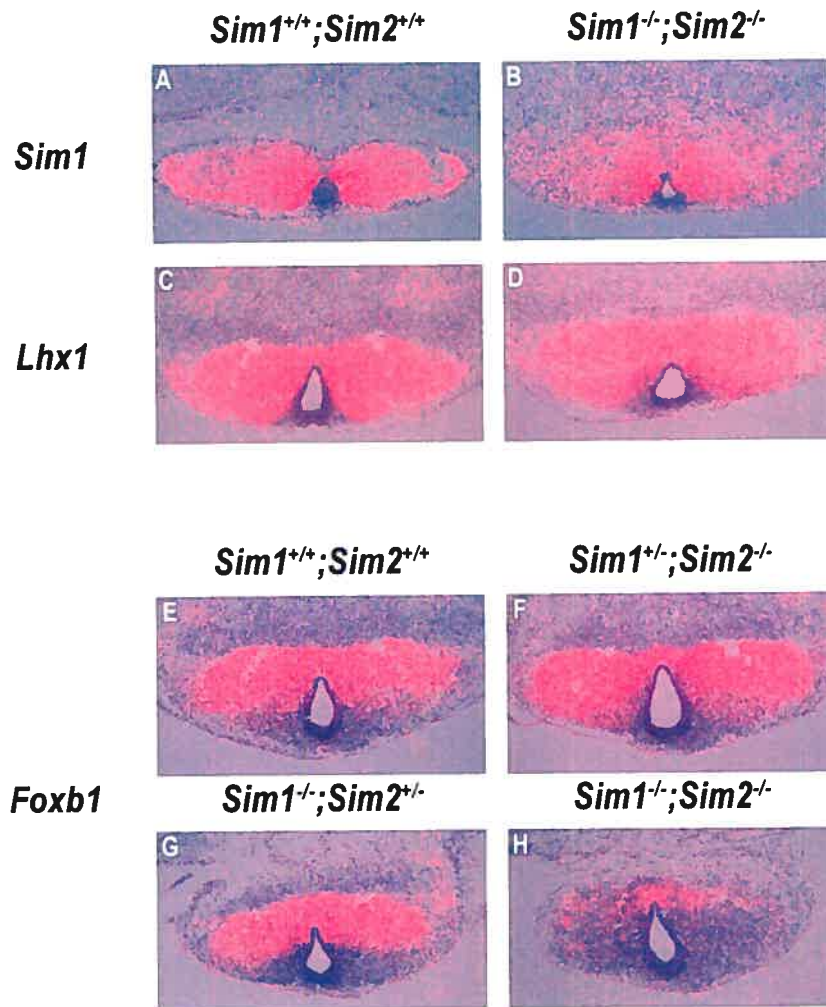
Figure 9.

Figure 9. *Mammillary body neurons survive until the end of gestation in Sim1/Sim2 double mutants.* Coronal sections through the MB of E18.5 with various dosage of *Sim1/Sim2*, as indicated, hybridized with *Sim1* (**A,B**), *Lhx1* (**C,D**) and *Foxb1* (**E-H**) probes. *Sim1* and *Lhx1* expression is maintained in MB of *Sim1/Sim2* double mutants. In contrast, *Foxb1* expression is dramatically reduced in *Sim1/Sim2* double mutants but not in embryos with at least one allele of *Sim1/Sim2*. Of note, the apparent smaller sizes of MBs shown in (**G**) and (**H**) reflect different plans of section.

Figure 10.

Figure 10. *Sim1 and Sim2 repress Rig-1 expression in the developing mammillary body.* (**A-T**) Coronal sections through the MB of E12.5 *Sim1$^{+/-}$;Sim2$^{+/+}$* and *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos were hybridized either with a *Slit1* (**A-D**), *Slit2* (**E-H**), *Robo1* (**I-L**), *Rig-1* (**M-P**) or *Sim1* (**Q-T**) probe. Sections correspond either to the anterior or posterior aspect of the MB, as indicated. The sections hybridized with the *Rig-1* probe (**M-P**) are adjacent to those hybridized with the *Sim1* probe (**Q-T**). Expression of *Slit1*, *Slit2* and *Robo1* is similar in *Sim1$^{+/-}$;Sim2$^{+/+}$* and *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos. In the anterior MB of *Sim1$^{+/-}$;Sim2$^{+/+}$* embryos, *Rig-1* is expressed in a narrow region which includes a medial domain (yellow arrowhead) that is contained within the dorsal aspect of the MB *Sim1* expression domain (**M,Q**). In *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos, *Rig-1* expression in the prospective MB (arrow) occupies a larger area extending ventrally and laterally (**N,R**). *Rig-1* expression in the region dorsal to the MB (blue arrowhead) is decreased in *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos (**M,N**). In the posterior MB, *Rig-1* expression is upregulated in *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos (arrow) (**O,P**). (**U-B'**) Coronal sections through the MB of E11.5 *Sim1$^{+/-}$;Sim2$^{+/+}$* and *Sim1$^{-/-}$;Sim2$^{-/-}$* embryos were hybridized either with a *Rig-1* (**U-X**) or *Sim1* (**Y-B'**) probe. Sections correspond either to the anterior or posterior aspect of the MB, as indicated. The sections hybridized with the *Rig-1* probe (**U-X**) are adjacent to those hybridized with the *Sim1* probe (**Y-B'**). *Rig-1* is ectopically expressed in the MB (arrows). Asterisks indicate a second region in which *Rig-1* expression is upregulated (**W,X**).

ANNEXE II: CHARATERIZATION OF REGULATORY ELEMENTS DRIVING SIM1 EXPRESSION IN THE BRAIN USING TRANSGENESIS

The basal promoter sequence of *Sim1* gene is contained within an 8.1kb genomic sequence named fragment N, extending upstream of exon2 (fig.1A). The ability of this fragment N to drive the expression of a LacZ reporter gene was tested by transgenesis in mice (fig. 2A). Analysis of three transgenic lines did not reveal LacZ expression in the central nervous system of E12.5 embryos and newborn mice. One explanation for the lack of LacZ staining in these animals would be that the transgenic construct contains repressor elements that silence a PVN specific element. In order to verify the existence of these potential elements, we studied the impact of deletions of fragment N using a transfection assay in cultured Neuro2A cells (N2A). Mouse neuroblastoma N2A cells are immortalized and express several markers of the PVN, including vasopressin, melanocortin receptor 4 (*Mc4r*), *Arnt2* and *Sim1* (Bamberger et al., 1995; Adan et al., 1996; Yang et al., 2004). Although it is difficult to conclude definitively that these cells are of hypothalamic origin, they appear to represent a reasonable model to dissect *Sim1* regulatory elements.

We generated series of constructs in which fragment N, or segments of fragment N were cloned upstream of a luciferase cDNA in a pGL3 promoterless vector. These constructs were transiently transfected in N2A cells, and their promoter activities were assayed. Fragment N did not induce luciferase activity, correlating with the results obtained with transgenic experiments. Interestingly, a short fragment, named A, induced a 6-fold increase of luciferase activity over baseline (fig. 1B), which suggests that a negative regulatory element exists in the 8.1kb of 5'-sequence. In parallel, we used a bio-informatic approach to identify potential regulatory regions in the *Sim1* locus. We compared human and mouse *Sim1* sequences over 10 kb of 5' sequence as well as in intronic regions. We identified two regions sharing high identity, one is located in fragment A, corresponding to the basal promoter, and another one is a 670 bp sequence located in the second intron that showed

a 90% identity (named fragment M). We found that fragment M itself doesn't have any promoter activity, but it increases promoter activity by 12.6 fold when associated with fragment A (M+A) (fig.1B).

We next verified whether combination of M+A could drive LacZ expression in the CNS of transgenic mice. The expression patterns of these transgenic embryos were analysed at the embryonic day 12.5; at this stage, endogenous *Sim1* expression is high in the brain. Expression of LacZ driven by M+A is detected in some domains of endogenous *Sim1*, such as mamillary body and midbrain but not in the PVN (fig. 2B). LacZ expression is subtle in these brain regions and shows variable patterns. Thus, the regulatory elements found in this transgene appear to be weak. The variability of LacZ patterns could be related to the influence of positional factors on such a weak transgene.

A

B
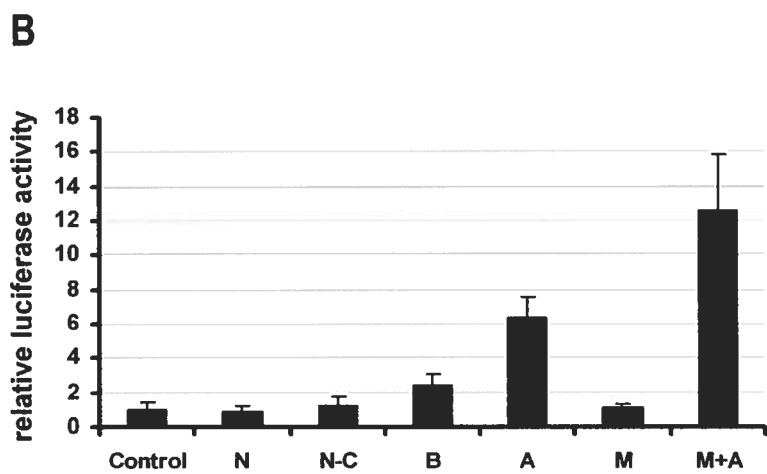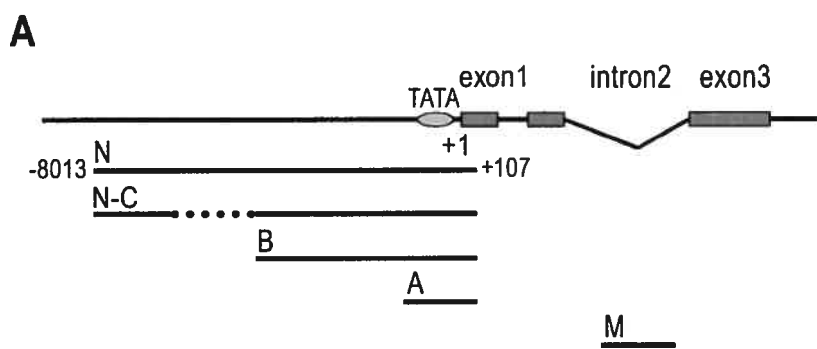
Figure 1.

Figure 1. Charaterization of *Sim1* regulatory elements in cultured cells.
(A) Schematic depiction of various sub-fragment of a 8.1kb 5' sequence of
Sim1 gene. N fragment is 8.1kb, from −8013 to +107; N-C fragment is the N
fragment lacking segment C which is from -4417 to -1980; B fragment is from
-1980 to +107; A fragment is from −556 to +107; M fragment is a 670bp
sequence located in intron 2 and conserved in human and mice. The promoter
region is indicated by a TATA box. **(B)** Promoter activity of *Sim1* genomic
fragments. Each of the indicated fragments was cloned upstream of a
luciferase reporter gene. The resulting construct was transiently transfected in
N2A cells and the luciferase activity was measured. Relative luciferase
activities are expressed as fold induction over the value obtained from
transfection of the pGL3 promoterless vector (control).

A

N ⬭ **LacZ** | PolyA

M+A ▬⬭ **LacZ** | PolyA

B midbrain
PVN
MB

C

D

E

F
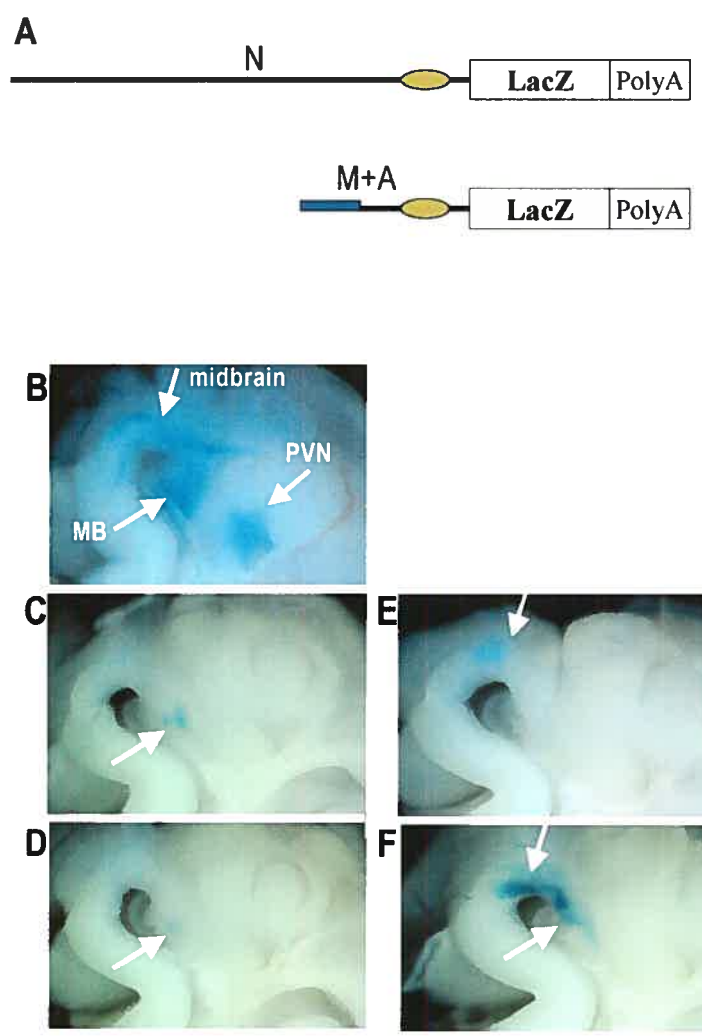
Figure 2

Figure 2. Charaterization of *Sim1* regulatory elements using transgenesis. **(A)** Schematic of transgenic constructs. *Sim1* promoter region is indicated by a TATA box. **(B)** Whole-mount in situ hybridization showing *Sim1* expression in the brain of E12.5 embryo. **(C-F)** LacZ expression in the brain of 4 out of 6 embryos that carry the transgene M+A. LacZ expression domains driven by the M+A sequence of the *Sim1* gene are pointed by an arrowhead. MB, Mammillary body; PVN, paraventricular nuclear.

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6. <u>Chun Yang,</u> Bin Yu, Dujin Zhou and Shiuan Chen. Regulation of aromatase promoter activity in human breast tissue by nuclear receptors EAR-2, EAR-3 (COUP-TF1), and RARγ. The 91st Annual meeting of American Association for Cancer Research. April 5, 2000, San Francisco, USA.

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