A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene.

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ABSTRACT

The onset of feeding at birth is a vital step for the adaptation of the neonate to extra-uterine life. Prader-Willi syndrome (PWS) is a complex neurogenetic disorder caused by the alteration of several imprinted contiguous genes including MAGEL2. PWS presents with various clinical manifestations, including poor suckling behaviour and feeding problems in neonates. Hypothalamic defects have been proposed but the pathophysiological mechanisms remain poorly understood. Here we report that a Magel2 deficient mouse with 50% neonatal mortality had an altered onset of suckling activity and subsequent impaired feeding, suggesting a role of MAGEL2 in the suckling deficit seen in PW newborns. The hypothalamus of Magel2 mutant neonates showed a significant reduction of oxytocin. Furthermore, injection of a specific oxytocin receptor antagonist in wild type neonates recapitulated the feeding deficiency seen in Magel2 mutants, and a single injection of oxytocin, three to five hours after birth, rescued the phenotype of Magel2 mutant pups, allowing all of them to survive. Our study illustrates the crucial role of feeding onset behaviour after birth. We propose that oxytocin supply might constitute a promising avenue for the treatment of feeding difficulties in PW neonates and potentially of other newborns with impaired feeding onset.
INTRODUCTION

After delivery, adaptation to extra-uterine life involves a set up of several vital processes including oral feeding. The transition is abrupt and needs swift appropriate behaviour. The mechanisms which trigger the onset of feeding at birth are still poorly understood.

Prader-Willi syndrome is a rare genetic disease (PWS; OMIM 176270) with a complex and progressive phenotype (1) including feeding problems from birth. PWS is viewed as having two identifiable phases in the feeding behaviour. During phase I of the syndrome, failure to thrive, feeding difficulties and slow weight gain as well as impaired growth are the most striking symptoms; newborns have poor muscle tone and the suckling activity is weak or absent. Tube feeding is often required in the first weeks of life and babies show little interest in feeding during the first few months of their lives. Paradoxically, during phase II (after 2 years approximately), an insatiable appetite takes over and children often demonstrate voracious food seeking behaviour (2). To date, much of the PWS phenotype is consistent with a hypothalamic dysfunction (3) but the pathophysiological mechanism is not yet completely identified. Noticeably, in some adult PW patients a reduction in the number of oxytocin-expressing neurons in the paraventricular (PVN) region (4) has been reported and an abnormally low level of circulating oxytocin (5).

Genetically PWS is a contiguous gene syndrome resulting from the lack of paternal expression of several imprinted genes located in the 15q11-q13 region; in healthy individuals as in PWS, the maternal copy of these genes is always silenced (1). Human genetic studies revealed three patients with different microdeletions (6,7,8), encompassing the SNORD116 genes, encoding for Small Nucleolar Orphan RNAs. These patients present common clinical features such as developmental delay, hyperphagia, obesity, hypogonadism and infantile hypotonia described as major PWS symptoms. Although infantile feeding problems are also
reported, only one patient had a reported significant hypotonia and weak suckling from birth requiring tube feeding (8). Consequently, a major role of the SNORD116 locus in the PWS phenotype is now supported. However, the question about the mechanism(s) by which these deletions, including the SNORD116 locus, cause the physiopathology observed in these patients remains open. Effectively, it might be through the functional role of SNORD116 SnoRNAs, which is poorly understood, and/or it might also involve an impairment of the expression of the other PWS candidate genes in the brain of these patients, PWS genes being highly expressed in the brain. The data available in the literature are not sufficient to discriminate these two alternatives.

Several murine models have been generated resulting in an absence of expression of all the paternally-expressed genes; they recapitulate the early failure to thrive and show perinatal lethality but none of surviving mice showed obesity (see for review (9)). Targeted mutations of specific genes (such as Necdin, Snord116) have been created and, depending on genetic background and on the level of investigation of these models, the phenotypes recall some of the PW features. For instance a role for NECDIN in PWS breathing deficits is strongly suggested (10,11). Two mouse models with a deletion of the Snord116 gene cluster have been reported (12,13). Both models result in postnatal growth retardation suggesting a role of SNORD116 RNAs in the failure to thrive observed in PW infants. One model was further investigated showing hyperphagia but no obesity, delayed sexual maturation, increased anxiety, defect in motor learning(13). One of the candidate genes in this region, MAGEL2, belongs to the family of MAGE (Melanoma Antigen Gene Expression) genes (14,15), the function of which is still elusive. In the mouse adult brain, Magel2 transcripts are expressed in several structures and in the hypothalamic nuclei a strong and circadian expression was detected (Allan brain Atlas, http://www.brain-map.org; http://expression.gnf.org; (16,17)). Magel2 is also strongly expressed in the mouse developing nervous system (18,19).
Previously, a mouse model for Magel2-deficiency has been created. Adult Magel2 deficient mice showed circadian dysfunction and reduced motor activity, impaired reproductive function, altered metabolism resulting in increased adiposity after weaning and behavioural problems (17,20,21,22). A postnatal lethality (around 10%) is observed (17) and neonatal growth retardation due to a reduced gain weight observed from birth until weaning (20) has also been described but the cause(s) has not been further investigated.

Here we describe a new mouse mutant deficient for Magel2 recalling the suckling deficit of human PW newborns and suggesting that Magel2 plays a role in the failure to thrive described in PWS. The present study reflects the vital importance of feeding onset in neonates and provides evidence for a pivotal role of Magel2 in this behaviour. Magel2 deficiency correlated with a significant hypothalamic decrease of oxytocin (OT). The use of a specific OT-receptor antagonist in wild type animals and oxytocin rescue experiments in Magel2 KO neonates demonstrated a relationship between oxytocin deficiency and poor feeding behaviour at birth. Importantly, our results lead us to propose a therapeutic treatment for the suckling deficit of PW neonates by external supply of oxytocin.
RESULTS

Neonatal death of Magel2 deficient mice

Gene targeting was used to generate Magel2-null mice on 129Sv/Pas genetic background (Fig.1 and Supplementary data). Since Magel2 is an imprinted gene with a paternal expression only, mice with a paternally deleted Magel2 allele (m+/p-) are thereafter referred as Magel2 KO mice since they are functionally equivalent to Magel2 null mice. Indeed Magel2 transcripts were not detected in Magel2 KO embryos (Fig.1d), compared with wild type individuals from the same litter.

Magel2 KO mice were significantly under represented at weaning time (Supplementary Table1). This under-representation was dependent on the genetic background, with a 51.4% lack of Magel2 KO mice after 5 to 7 backcrosses on C57Bl6/J genetic background (Table1b). In the surviving mutants, males and females were equally represented.

During embryogenesis (from E10.5 to E18.5) and in early neonates (2-4 hours old), 50% of Magel2 deficient mice were observed, as expected in a Mendelian ratio (Supplementary Table 2). However, genotyping of litters at P1, revealed that only 58% of Magel2 KO mutants versus 99% of wild type (Supplementary Table 2) were alive whereas 42% of Magel2 KO mutants versus 1% of wild type (WT: n=1, KO: n=32; CHI² test, P<0.001) died by the end of P1 (carcasses being present in the box). All the studies reported thereafter were done on mutant or wild type pups after 7 backcrosses on a C57Bl6/J genetic background (over 98%).

Feeding deficiency in Magel2 mutants pups: the cause of neonatal death.

Newborn Magel2 mutants displayed normal external appearance, and normal pink-colored skin indicating proper circulatory and respiratory functions. Consistently, the hearts of mutant
neonates beat normally. All organs were present in the KO neonates and looked similar to those of wild type littermates as confirmed by histological analyses (Supplementary Fig.1). However, in a population of 26 litters (104 WT and 104 KO) analysed 3 to 8 hours after birth, 56% of Magel2 KO newborns had no milk in their stomachs compared to 16% of the controls (CHI²=116, P<0.0005). Approximately 12 hours after birth, in Magel2 KO surviving newborns (25% were dead), the level of glycaemia was significantly reduced (less 30%) in mutant compared to wild type neonates (WT: 55 mg/dl (46, 65), n= 42; KO: 39 mg/dl (28, 52.5), n=35; Wilcoxon-Mann-Whitney (WMW) test, P<0.001). All these data suggested a problem of feeding in Magel2 KO newborns. Indeed, in ten novel litters, 58% (19/39) of Magel2 KO individuals died by the end of P1 and post mortem analysis revealed that none of them had milk in their stomach.

In parallel, we followed the growth of surviving mutants compared to wild type newborns. First, between P0 and P3, we observed a 43% significant reduction in the weight gain of Magel2 KO compared to wild type individuals (4 litters, WT: 0.52 g (0.27, 0.73), n= 16; KO: 0.22 g (0.18, 0.31), n=13; WMW test with stratum=litter, P< 0.005 ); but this difference in weight gain disappeared between P3 and P4 (WT: 0.33g (0.34, 0.35), n= 16; KO: 0.34 g (0.34, 0.39), n=13, WMW test N.S.). Until weaning, these surviving mutants displayed a tendency to lower mean body weight (approximately 10% lower) than the wild type animals; obesity was not developed in adulthood.

Taken together, these data suggest that in the absence of Magel2, the initiation of feeding process after birth is altered, leading to an early death of approximately 50% of mutants.

**Feeding behaviour in Magel2 KO pups: the onset of suckling activity is disturbed.**

One crucial step to survive is to find food. Indeed, mouse neonates have to find their mother’s nipples and suckle by themselves. This step involves: an awakened state, an olfactory or
tactile functional system to locate the mother’s nipples, a rooting reflex, a rhythmic suckling reflex and swallowing (23). Thus, several sensory and motor systems involving different brain structures and muscles are implicated (24).

Soon after birth, Magel2 KO pups did not appear hypotonic and reacted with similar movements as observed in controls, notably when they are placed on their back. During the first postnatal day, control and mutant pups exhibited a similar rooting reflex (Supplementary methods). Furthermore Magel2 KO newborns could open and close their mouth normally. All these observations are in agreement with a normal motor activity necessary for suckling in Magel2 mutants. Importantly, all mutant newborns are together with their littermates, close to the mother at least during the 12 first hours and later for surviving animals, suggesting that separation and lack of mother care is not involved in the phenotype of mutant newborns.

Then, we performed a suckling test, recording the attachment of the newborn to the mother’s nipple (25). We observed a significant difference in suckling activity (WMW test, P<0.005) between wild type (n=43) and Magel2 KO (n=31) newborns issued from 10 litters (Table 1): 39% of Magel2 mutant versus 2% of wild type animals had no suckling activity, 39% of Magel2 mutant versus 21% of wild type animals had a weak suckling activity and only 22% of Magel2 mutant versus 77% of wild type animals have a strong activity. Altogether, these data suggested that at birth Magel2 KO mice had defects in the initiation of suckling stimulation leading to impaired feeding and death of ≈ 50% of newborns. The remaining mutant neonates were able to initiate the suckling process and to survive.

**Neuroendocrine function during the early postnatal period in Magel2 KO**

Magel2 is strongly expressed in the hypothalamus that is crucial for feeding control. Moreover hypothalamic dysfunction is suspected in the pathophysiology of PWS (for review (3)). There was an absence of obvious structural modifications in brain anatomy and
particularly in the hypothalamus of mutant newborns (Supplementary data and Fig.2). This result did not exclude a functional alteration. The production of neuropeptides and hormones might be modified, in particular those which are known to play an important role at birth. Circulating glucocorticoids play an important role in the adaptation of the neonate to the extrauterine life (26,27), in particular in the regulation of energy homeostasis (28). Just after birth, a sex independent abrupt rise in plasma AdrenoCorticoTropin Hormone (ACTH) (10 min after birth) has been previously described, followed by an increase in plasma and adrenal corticosterone levels (one hour after birth) (26). This de novo synthesis might be involved in the initiation of primitive reflexes. We then used enzyme immunoassay (EIA) tests and compared the level of plasma ACTH, in caesarean-section newborns from pregnant females at term (E18.5); we did not observe any significant difference in the ACTH plasma level between Magel2 KO and wild type mice issued from three litters (WT: 5.4 ng/ml (4.6, 5.8), n= 9; KO: 5.9 ng/ml (5.4, 6.1), n=11; WMW test, N.S.). Using EIA, we also quantified and compared the level of plasma corticosterone, 60 min after birth. There was no significant difference between both genotypes, male newborns being issued from 5 litters (WT: 750 ng/ml (650, 1050), n= 10; KO: 575 ng/ml (425, 750), n=10; WMW test, N.S.).

Several hypothalamic neuropeptides such as Oxytocin (OT) (29), Arginine-Vasopressin (AVP) (30,31,32), and Orexin-A (OXA) (33) are also thought to play an important role during the early postnatal period, however their role in onset of feeding at birth is not clear. Using an EIA approach, the quantity of these mature neuropeptides produced in the hypothalamus and in the hypophysis of Magel2 mutant neonates and their wild type littermates was measured. In the hypothalamus, we observed an average 36% reduction of mature OT, a 22% reduction of mature OXA and a 20% reduction of mature AVP (Table 2). The hypothalamic levels of ACTH and of alpha-Melanocyte Stimulating Hormone (α-MSH) which are two products derived from propiomelanocortin did not differ between mutants and wild type newborns
(Table 2). In the pituitary gland, the quantity of OT, AVP and ACTH was not significantly different between both genotypes (Table 2). In conclusion, the deficiency in *Magel2* expression led to an impairment of hypothalamic production of at least several mature amidated neuropeptides (OT, AVP, OXA), the production of OT being the most affected.

**Immunohistochemical analyses of hypothalamic neurons producing OT, AVP and OXA.**

The hypothalamic neurons expressing these peptides (OT, AVP, OXA) were studied by immunohistochemistry. Stronger OT immunolabelling was observed, 3-5 hours after birth, in the PVN of mutant compared to wild type animals (3 mice per group), using a first antibody against OT that did not discriminate between the different OT forms produced in its maturation process. This observation did not seem consistent with the 36% decrease in OT mature form seen in *Magel2* KO hypothalamus by EIA (see above), but might be explained by an accumulation of not fully processed OT forms. In order to discriminate which forms might be accumulated, we then used two antibodies allowing us to specifically stain for 1) OT-associated neurophysin (PS-38) detecting the respective OT prohormone and 2) for the OT-peptide intermediates (VA-10) (34,35,36). Using co-immunolabelling (Fig.2a) followed by confocal microscopy analysis, we quantified the number of positive cells for each antibody in the para ventricular nucleus (PVN) of newborns (3-5 hours after birth). The number of PS-38 positive cells was similar between mutant and wild type animals (WT: 237 (226, 298), n=5; KO: 300 (279, 303), n=7; WMW test, N.S.). But, on the same sections, the number of VA-10 positive cells was significantly higher in the PVN of *Magel2* KO compared to wild type PVN (WT: 259 (239, 309), n=5; KO: 442 (387, 469), n=7; WMW test, P<0.005). Since the VA-10 antibody recognizes specifically intermediate OT forms (mainly OT-G), we conclude to a 1.7 fold increase in the detection of intermediate OT forms in the *Magel2* KO PVN compared to wild type PVN.
Similarly to this study, we performed a co-immunolabelling on PVN sections using antibodies specifically raised against the AVP-associated neurophysin (PS-41) and the AVP-peptide intermediates (VA4) (34,35,36). A confocal analysis (Fig.2b) and quantification of PS-41 (KO: 375 (304, 386), n=6; WT: 362 (314, 387), n=6; N.S.) and VA4 (KO: 379 (360, 391), n=6; WT: 407 (390, 417), n=6; WMW test, N.S.) positive neurons in mutant and wild type PVN revealed no significant difference between both genotypes. Thus, in Magel2 KO PVN, we did not detect an increase of intermediate AVP forms as we observed for OT.

Finally, we also used an antibody that recognizes specifically the prepro-orexin, the initial unprocessed form of OXA. We observed a similar immunolabelling and we counted the same number of prepro-orexin-expressing neurons in the lateral hypothalamus of mutant (258 (253, 269), n=5) and wild type (264 (235, 266), n=5) animals analysed at P0 (data not shown).

In conclusion, these results revealed a specific accumulation of OT intermediate forms in Magel2 KO compared to wild type neonates hypothalami although the OT-prohormone is similarly expressed. One interpretation is that the transformation of OT intermediate forms to amidated OT is impaired in Magel2 KO hypothalami in neonates, leading to an accumulation of these intermediate forms and to a reduced quantity of mature amidated OT. Noticeably, the fully processed amidated form of OT is detected one day before birth and increases abruptly after birth (35,36). Thus, another interpretation is that the maturation of OT neurons is delayed in mutant hypothalami leading to an accumulation of OT intermediate forms, hence an impairment in the release of mature OT, normally observed at birth.

**Injection of an oxytocin receptor antagonist prevents feeding in wild type neonates and induces postnatal lethality**

Altogether, the previous data, suggested that OT deficiency might participate in the Magel2 KO feeding phenotype at birth. To validate this hypothesis, the SSR 126768A compound, a
specific OT antagonist (OTA) that displays even higher affinity (6-fold increase) for the OT receptor than natural OT and a low affinity for AVP-receptors (37), was used in wild type animals. We proceeded to a single injection of 3µg (20µl) (37) in 68 wild-type C57Bl6/J newborns (9 litters), 1 to 1.5 hour after birth. In parallel, in the same conditions, we injected the vehicle (20µl) in 48 wild-type C57Bl6/J newborns (7 litters). Then, 12 (+/- 3) hours after the injection time, we observed than 54% (37/68) of newborns injected with OTA had no milk in their stomach versus 4% (2/48) of newborns injected with vehicle. At P1 (24-36h old), 48% (33/68) of newborns injected with OTA were dead versus 4% (2/48) of newborns injected with vehicle control. We concluded that the SSR 126768A OTA had a significant effect in wild type C57Bl/6J newborns, preventing half of them feeding correctly and leading to lethality at P1. However, when the SSR 126768A OTA (3µg in 20µl) was injected in 42 wild-type pups, 12-24 hours after birth, all of them survived 48 hours later and appeared healthy. Thus, the SSR 126768A OTA at a dose of 3µg appeared to have a specific effect, preventing the feeding process and leading to death, only when it was injected in a specific window of time after birth, before the suckling activity was initiated. This phenotype simulated the Magel2 KO feeding phenotype at birth.

The suckling initiation deficit phenotype and the lethality resulting from a lack of Magel2 can be rescued by injection of oxytocin

The results obtained with the injection of SSR 126768A OTA allowed us to hypothesize that we might restore a normal feeding behaviour in Magel2 KO newborns with an OT injection just after birth. Early neonatal manipulation of OT was reported in the literature without alterations of the postnatal development (38,39,40). We then decided to inject 2µg of OT in Magel2 KO and wild type newborns, 3 to 5 hours after birth, the time when wild type newborns started to suckle but not the mutant neonates. In a first step, using two cohorts of
wild type pups (n=28), we checked that a subcutaneous (s.c.) injection of isotonic saline (20µl NaCl 0.9%) and a s.c. injection of oxytocin (2µg in 20µl of NaCl 0.9%) solution did not induce a specific lethality. In a second step, thirteen litters including a total of 49 wild type and 51 Magel2 KO neonates, were injected with an oxytocin solution. At P2, we observed that 96% (47/49) of wild type and 94% (48/51) of Magel2 mutant newborns survived with milk previously detected in their stomach at P1. In parallel, we controlled that the rescue of Magel2 KO neonates was specifically due to the oxytocin injection and not to the injection stress and/or handling of neonates. In a control experiment we performed an s.c. vehicle injection in 33 Magel2 mutant and 37 wild type neonates. At P1, lethality among Magel2 KO individuals was at 48.5% (16/33) while only one wild type pup died. Noticeably the death of these 16 Magel2 mutants correlated with a lack of milk in the stomach of the carcasses. The high percentage (48.5%) of lethality observed in Magel2 pups but not in wild type pups, both being injected with isotonic saline, was comparable with the lethality observed in non treated Magel2 newborns.

Similar experiments were performed with AVP injections (Supplementary data) but whatever the dose injected, AVP did not rescue the phenotype of Magel2 deficient neonates. We conclude that one single injection of OT, in a specific window of time, 3-5 hours after birth, was able to efficiently rescue the death rate due to the feeding deficit observed in Magel2 KO newborns.
DISCUSSION

The mechanisms that trigger the onset of feeding at birth are complex and multifactorial. The cause of feeding problems encountered in PW newborns is often viewed as a consequence of the underlying hypotonia that in turn would result in poor suckling, but this has not been proven and alternative explanations are possible. The inability to suckle properly is also met with a lack of interest in food and/or avoidance behaviour. Here, we show that the Magel2 KO neonates, while not hypotonic, are unable to feed, as a consequence of their inability to search food and to suckle properly. This study demonstrates for the first time a role of Magel2 in the onset of feeding. Magel2 KO mouse is the only model, with the abrogation of one specific gene among all PW candidate genes leading to a deficit in suckling initiation. We propose that the lack of MAGEL2 contributes to the suckling deficit and, consequently, to the failure to thrive observed in PW infants (see for review (41,42)). In this line, it is noticeable that a mouse model in which Mkrn3, Necdin, Magel2 (43) showed no expression, is associated with neonatal lethality (depending on the content of C57BL/6J genetic background) suggestive of a failure to thrive although mice lacking the paternal expression of Mkrn3 (44) or Necdin do not present a failure to thrive at birth. Furthermore, one patient with a deletion including MKRN3, MAGEL2 and NECDIN (45) had no PWS but some symptoms with feeding problems during her first 18 months of life.

The failure to thrive described in PWS is probably caused by a complex mechanism involving at least two genes, MAGEL2 and SNORD116 gene cluster. Effectively, human genetic studies revealed a role of the SNORD 116 gene locus in the failure to thrive in PW infants although the question of a direct functional role of the SNORD 116 SnoRNAs remains open (see introduction). In addition, both Snord116 knock-out mouse models showed a growth deficiency. Apparently, this growth deficiency does not result from a deficit of the suckling
initiation (as in Magel2 KO neonates) because, firstly, the phenotype appears at P2 (13) or P5 (12) but not from birth and, secondly, the presence of milk was detected in the stomach of P5 neonates (13). Furthermore, Ding et al. (42) showed that the gene expression profiles of Snord116 deletion mice and wild-type littermates were very similar at all time points and conditions, arguing against a role of Snord116 in feeding regulation in the neonatal period. Taking into account all these data, we conclude that SNORD116 gene cluster and MAGEL2 might contribute together to the failure to thrive observed in PWS. But, in this process, a different role might be attributed to each locus: MAGEL2 being an actor of the suckling initiation and SNORD116 locus playing a role in the postnatal growth, with a yet unidentified mechanism.

The rescue of the present Magel2 KO phenotype via oxytocin injection has potentially important consequences and applications in the therapy/management of PW newborns. The forced, long-lasting and invasive feeding procedure required in PW infants might be alleviated by a unique oxytocin supply, which would trigger normal feeding onset at birth and subsequent maintenance of feeding behaviour.

In the previous reported Magel2 KO mouse model, a postnatal lethality (10%) (17) and a neonatal growth retardation (20) have been reported but not further studied. Thus, a similar deficit in the feeding behaviour might exist in this Magel2 KO strain but has not been described because it has not been further investigated. The authors described mainly the adult phenotype of Magel2 KO mice and, interestingly, we observed similar deficiencies (circadian rhythm, infertility and behaviour problems) in surviving mutants of our Magel2 KO model (F.M., personal data). This suggests that, indeed, the phenotype might be similar between both KO mouse models with variability in its expression leading to a different rate of lethality. Such variability might be a consequence of a difference in genetic background between the mouse strains. Similarly, a strong difference in the rate of lethality has been observed for the
Necdin KO mouse models (46,47) but the cause of death was the same, a breathing deficiency (48). Finally, a difference in the KO molecular strategy is noticeable since the promoter has been deleted in our KO mouse model but it has been kept in the previous model to allow the expression of LacZ gene. The presence or absence of the promoter might change the severity of the phenotype by modifying the expression of proximal genes that would share regulatory elements or by modifying the expression of other genes from the MAGE gene family that have identical regulatory sequences.

The role of hypothalamic circuits and of different neuropeptides in controlling food intake, body weight and glucose homeostasis is crucial and well documented in mature mammals (49) as in the postnatal period (50,51). However, the role of the hypothalamus in the on set of oral feeding at birth has been barely investigated. In Magel2 mutant pups, the organization of hypothalamic nuclei was apparently normal. However a significantly reduced amount (36%) of amidated mature OT in Magel2 KO hypothalami was observed as compared with wild type hypothalami of neonates. Our data also showed that the decreased amount of OT correlated with an accumulation of C-terminally unaminated intermediate forms of OT although, in the same cells, the prohormone (Pro-OT) showed normal expression. Interestingly, during fetal life and even in early neonatal brain, the Pro-OT precursor is incompletely processed to unamidated forms of OT (36). This delay in posttranslational processing of the OT peptides appears to be specific of OT neurons. In the absence of Magel2 expression, such an accumulation of partially processed forms of OT might result either from a direct alteration of a process involved in the maturation of OT intermediate forms into amidated forms, or from a delay in the maturation of OT neurons, such delay would indirectly prevent a normal release of amidated-OT at birth. As the cellular and molecular functions of Magel2 remain unknown to date, the mechanism underlying this deficiency of amidated OT production and its link with Magel2 deficiency cannot be explained yet. In the same Magel2 KO hypothalami, we also
observed a significant reduction (20%) of AVP and OXA production. Immunohistochemistry analysis did not reveal an accumulation of intermediate AVP forms as we observed for OT. However, the number of cells expressing the AVP prohormone (NP-AVP) and intermediate AVP forms in the PVN being similar in mutant and wild type, we cannot exclude that an impairment in prohormone processing does also exist for AVP leading to a 20% decrease of production of mature forms. Interestingly, previous studies on five post-mortem adult PWS brains using IHC (52,53) suggest a processing defect in AVP production in PW patients. Similarly, the number of cells expressing OXA prohormone (prepro-orexin, data not shown) in the lateral hypothalamus is the same between mutant and wild type pups although a reduced OXA quantity is detected in Magel2 KO animals. In this line, Kozlov and his colleagues have suggested that, in adult hypothalamus of their Magel2 KO mouse model, the maturation process of prepro-orexin was altered (17).

Whatever the mechanism involved, we show here that Magel2 plays a crucial role in the onset of feeding at birth. We also showed that the OT/OT receptor system might be a major stimulus for the newborn to initiate milk intake. Interestingly, the injection of a specific antagonist of the oxytocin receptor (SSR 126768A) in wild type newborns after they had already ingested milk, had no more effect on survival and on feeding behaviour in the following days, as opposed with the effects of such an injection before feeding onset. Thus, this study reveals an as-yet unknown and unexpected role of OT in the initiation of feeding behaviour, in a specific time-window just after birth. Does this mean that oxytocin, produced by the newborn, is a factor necessary to trigger the onset of feeding? Noticeably, in rats, the fully processed amidated form of OT is detected one day before birth and increases abruptly after birth (35,36) although its function at this time is not known. Furthermore, a study on rabbit pups (54) reported that the oxytocinergic system of the supra optic nucleus and PVN is differentially activated in pups by suckling of milk or by anogenital stroking, a sensory
stimulation triggering suckling activity. These data support a role of OT, at birth, in stimulating the search of food and/or in initiating the suckling activity. Surprisingly, the mice knock-out for the gene encoding OT are viable and no feeding problem has been reported at birth (55,56,57). In those animals, alternative pathways may be called in action and substitute for the fully defective OT production. For instance the endocannabinoid–cannabinoid receptor system has also been shown to play a role in the initiation of the milk suckling process during the first days postnatally in the mouse (58). In contrast, Magel2 KO mice have ‘less effective only’ OT system than wild-type animals and have also a significant reduction of hypothalamic OXA and AVP; for still unexplained reason, such or other alternative pathways might be operating in only the approximately 50% of Magel2 mutants that are able to feed and survive.

In conclusion, the present study provides the first evidence for a key role played by Magel2 and by the OT/OTR system in the initiation/stimulation of feeding behaviour at birth. These results open a novel therapeutic strategic option in the impairment of onset feeding behaviour observed in PW newborns. Moreover, feeding is often disturbed in babies with various genetic syndromes and in infants presenting with an inability to ingest food and known as “non-organic failure to thrive” syndrome (2). Administration of OT might be a safe and powerful therapeutic agent not only in PWS but generally to stimulate the feeding behaviour and milk ingestion in those various feeding disorders with early onset.
MATERIALS AND METHODS

Ethics statement

All breedings and experiments were carried out in keeping with the European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC).

Mouse Magel2 KO line generation

Two fragments containing genomic sequences 5’ and 3’ to the Magel2 region to be deleted were amplified by PCR from 129Sv/Pas DNA and further verified by sequencing. The 2.651 kb XhoI and 3.062 kb NotI fragments, localised respectively 372 and 3062 bp 5’ and 3’ to the transcriptional initiation site of the NT-039428.7 Magel2 mRNA (an intronless gene), were inserted on each side of a LoxP-pgk1-hygromycin-LoxP cassette subcloned into a pBluescript II-SK vector (Stratagene). ES (129Sv/Pas genetic background) were electroporated and 240 ES cells resistant to hygromycin were screened for homologous recombination and monoinsertion events by Southern blot analysis. Selected clones were electroporated with a Cre recombinase expressing vector. Seven ES cells clones carrying an allele in which the hygromycin resistance gene was deleted were selected by PCR, using primers 5’-CCCTGGGTTGACTGACTCAT-3’ and 5’-TCTTCTTCCTGGTGGCTTTG-3’, generating a 376 bp PCR product. Two clones were injected into C57BL/6J blastocysts. Chimeric animals obtained from one of these two clones allowed the transmission of the mutant Magel2 allele detected by PCR in agouti pups, using the same primers as those used to detect the deletion of the hygromycin resistance gene. Mice genotyping was performed by PCR on genomic DNA prepared from tail or placenta biopsies, using the same primers. The Magel2-KO colony was maintained in INMED animal facility (Marseilles). All the studies were done on mutant or wild type pups on a C57Bl6/J genetic background over 98% (after 7 backcrosses).

Neonates suckling behaviour
This test was performed as previously described (25). Briefly, 2-6 h after delivering their newborns, females were anesthetized with 0.1% Rompun Xylasine / 10mg/ml Ketamine Imalgene (0.1ml /10g of tissue) and laid down on their back, on a heated surgical pad (37°C). Newborns were individually tested for their capacity to find and grab to one of their mother’s nipple and to suck milk efficiently. Scores are described in Table1.

**In situ hybridization and Immunohistochemistry**

Tissues from whole embryos (E12.5) or P0 brains were dissected, fixed and treated as previously described (47,59). 14 µm sagittal or coronal frozen sections were collected. For Magel2 in situ hybridization (ISH) analyses, sections were treated and hybridized with a Magel2 antisens digoxigenin-labeled riboprobe (nucleotides 3366-4053; XM_622091), at 70°C in 50% formamide, as described previously (59). For double Magel2 and Necdin ISH analyses, sections were hybridized with Magel2 antisens digoxigenin and Necdin (nucleotides 2130-2420; D76440) fluorescein-labeled riboprobes (18). Immunohistochemistry on cryosections was performed as previously described (18). A polyclonal rabbit anti-Necdin (#07-565, Upstate; 1/500) was used and immunolabeling was detected with the goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Invitrogen; 1/500) as secondary antibody. Images were acquired with an Axiocam MRm digital camera (Zeiss) on an Axioplan2 Imaging microscope (Zeiss) with an Apotome module (Zeiss), using AxioVision 4.4.1.0 software (Zeiss). Images were assembled with the Adobe Photoshop CS2 software.

**Counting oxytocin, vasopressin and prepro-orexin neurons in P0 brains**

For oxytocin and vasopressin neurons counting, P0 brains (2-3 hours after birth) were dissected, fixed in AntigenFix (Diapath, Ref P0014) medium O.N. and included in agar 4%. 100µm vibratome coronal sections were collected from the anterior commissure to the posterior extremity of the hypothalamus. For oxytocin, initially we used a validated polyclonal anti-oxytocin (OT), which recognizes “unidentified forms” of OT (a gift from G.
Tramu (47)). Then, we performed co-immunolabelling using a mouse monoclonal antibody highly specific against the mouse neurophysin associated to OT(PS38) and a polyclonal antibody which recognizes the intermediate forms of OT(VA10) (34,35). For vasopressin, we performed co-immunolabelling using a mouse monoclonal antibody highly specific against the mouse neurophysin associated to AVP(PS41) and a polyclonal antibody which recognizes the intermediate forms of AVP (VA4) (34,35). PS38, PS41, VA10 and VA4 are a gift from Pr. H. Gainer.

For prepro-orexin neuron counting, P0 brains were treated as described in immunohistochemistry. 14 \( \mu \)m coronal sections were made with a cryostat and thaw-mounted onto sets of 5 slides, each slide containing rostro-caudal series of sections taken at 70 \( \mu \)m intervals. Sections were collected in the same region as above, which represented a mean of 24 sections. Individual set of slides for each P0 brains were stained with polyclonal rabbit anti-prepro-orexin (#AB3096, Chemicon; 1/250). Immunolabeling was detected with the following secondary antibodies: goat anti-rabbit Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes, Invitrogen; 1/500), goat anti-mouse Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes, Invitrogen; 1/500). Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Inc).

Images were acquired using a confocal microscope (Zeiss, LSM510), z stack of 6\( \mu \)m were performed for each image. Quantifications were performed with the image analysis software eCELLence (Glance Vision Technologies Srl). All positive neurons were counted on each section in order to count all positive neurons in the entire PVN (OT and AVP) or in the lateral hypothalamus (prepro-orexin).

**Enzymes-linked immunoassay analyses**

Enzyme-linked immunoassays analyses were performed to quantify levels of neuropeptides in P0 hypothalamus and hypophyses. P0 hypothalamus and hypophyses were dissected from
10:00 to 12:00 am (delivery occurring in early morning), before post-natal death affecting a proportion of *Magel2*-KO neonates occurred (mostly from 12:00 hours after birth to P1). Tissues were sonicated in a protease inhibitor cocktail (Complete Mini, Roche), incubated at 95°C for 10 min to completely inactivate proteases and kept on ice. The protein concentrations were determined on an aliquot of each sample. Neuropeptides were extracted by addition of HCl and acetic acid to a final concentration of 0.02 M and 0.1 M respectively. Samples were centrifuged at 5500 rpm for 20 min and the supernatants were aliquoted before being stored at -80°C. Concentrations of neuropeptides were determined by enzyme-linked immunoassays (EIAs) using Orexin A, Vasopressin[Arg8], Oxytocin, Agouti-related protein (AGRP), alpha-Melanocyte Stimulating Hormone (alpha-MSH), Adrenocorticotropic Hormone ACTH and corticosterone EIA Kits (Phoenix Pharmaceuticals, Inc) according to the manufacturer ‘s instructions. All samples, for series in which concentrations were to be compared, were extracted and assayed in the same experiment.

**Injection of Oxytocin and Vasopressin**

Three to 5 hours after delivery, wild type pups or pups issued from a cross between an heterozygote (+/-) male with wild type females were temporally removed (10 min) from their mother, weighted and given a single s.c. (in the ventral region at the level of floating ribs) injection (20µl) of isotonic saline or 2µg oxytocin (Phoenix Pharmaceuticals, Inc., Catalog No.051-01) dissolved in isotonic saline (20µl). This dose of OT was used because there is a literature indicating that during the neonatal period these doses are not toxic but can affect a variety of physiological responses in male and female rats and prairie voles as well as neuronal activation in neonates (38,39). Following the same protocol, we injected vasopressin (Phoenix Pharmaceuticals, Inc., Catalog No.065-07) at different doses. At P2, pups were scarified to be genotyped.

**Injection of SSR126768A**
A first cohort of C57Bl6 wild type newborns were removed from their mothers 1-1h30 after their birth and very rapidly were given a single s.c. injection (20µl) of vehicle control (0.2% ethanol in distilled water) or 3 µg (in 20µl of 0.2% ethanol in distilled water) of SSR126768A (OTA). A first cohort of C57Bl6 wild type newborns were injected 12-24 hours after birth. The dose of OTA used was based on previous publications (37).

We chose to use the Oxytocin antagonist SSR126768A (a gift from C. Serradeil-Le Gal, Sanofi-Synthélabo Recherche, Toulouse, France) because it was already well characterized. It was shown to have a high affinity for OTR in different species, including mouse, and a high selectivity for OTR with weak affinity for AVP receptors (37). It has been used at 1, 3 or 10 µg/g in rats (37) without inducing lethality or any strong secondary effect. Furthermore, at least on uterus, SSR126768A showed a rapid onset of action and a long duration effect (24h hours after administration with a dose as low as 3µg/g) (37). Then, we chose to use it at 3 µg/g in our experiments.

Statistical analyses

Taking into account the size of the sample and the normality or absence of normality of the distribution of the different values, we used appropriate statistical tools. Nonparametric statistical tools (Sigmastat software) or exact statistical tools (StatXact software) were used depending on the size of the sample (n). All tests are two-tailed tests. In the results, values are indicated as following: (Q2 (Q1, Q3), n, P value) where Q2 is the median, Q1 is the first quartile and Q3 is the second quartile. Wilcoxon-Mann-Whitney test is referred as WMW in the text. The level of significance was set at a P-value less than 0.05.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.
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V1a but not V1b receptors, and is independent of intracellular calcium signalling. *Eur J. Neurosci.* 24, 1565-1574.


FIGURE LEGENDS

Figure 1: Targeted Disruption of the Magel2 Gene

(a) Maps of the Magel2 wild type, the targeting construct and of the resulting mutant alleles, indicating the replacement of 3.397 kb region including the Magel2 promoter region and part of the Magel2 gene by a LoxP-Pgk1-hygro-LoxP cassette (hygro) placed in the opposite transcriptional orientation and introducing new EcoRV sites. The black box represents the Magel2 gene comprised by a single exon, the open box and the two open circles represent the hygromycin resistance gene under the control of the Pgk1 promoter, surrounded by the two LoxP sequences (LoxP-hygro-LoxP). The hygromycin resistance gene was secondary removed by expressing the Cre recombinase in ES cells clones, generating the Magel2- null excised allele. The probe used to identify homologous recombination in ES cells by Southern blot analysis is indicated as probe 1.

(b) Southern blot analysis of EcoRV-digested genomic DNA derived from hygromycin-resistant ES cell clones, allowing detection of both mutated (4.5 kb) and wild type (12.8 kb) Magel2 alleles.

(c) PCR analysis of ES cell clones electroporated with a Cre recombinase expressing vector. Deletion of the hygromycin resistance gene detected by the amplification of a 376 bp product is shown in the upper panel (the primers used for the PCR are represented by the two arrows surrounding the hygromycin cassette). It should be noted that the wild type allele cannot be detected as the fragment amplified is too long. A control PCR amplifying a Necdin genomic fragment is shown in the lower panel.

(d) Necdin and Magel2 in situ hybridization on transverse serial sections through the diencephalon region of E12.5 WT and Magel2 KO mouse embryos. III, third ventricule; hp,
hypothalamus region; lv, lateral ventricule; me, median eminence; vt, ventral thalamus; zi, zona intrathalamica. Scale bar: 185 m.

**Figure 2: Impairment in OT but not AVP maturation process in the *Magel2* KO PVN.**

On hypothalamic vibratome sections (100µm) covering the entire PVN of *Magel2* KO (n=7) and wild type (n=5) newborns, we performed a co-immunolabelling using PS-38 and VA-10 antibodies to reveal respectively OT-prohormone and OT-peptide intermediates (a). Similar experiment was done using PS-41 and VA-4 antibodies to reveal respectively AVP-prohormone and AVP-peptide intermediates on PVN of *Magel2* KO (n=6) and wild type (n=6) newborns (b).

In *Magel2* KO PVN, compared to wild type PVN, we did observe an accumulation of intermediate OT forms (a) but not of intermediate AVP forms (b).
Tables

Table 1: Suckling test

<table>
<thead>
<tr>
<th>Genotype of newborns</th>
<th>Suckling activity</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Weak</td>
</tr>
<tr>
<td>*WT</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>*KO</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>°WT</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>°KO</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

Suckling activity is considered as: a) absent if there is no attachment to the nipple (scored 0), b) weak if the latency for attachment is over 3 min and suckling activity itself is null or weak and not maintained (scored 1), or c) strong if the latency for attachment is less than 3 min and suckling activity itself is strong (scored 2).

*Pups tested are issued from three litters, about 2-3 hours old. Pups had no milk in their stomach before the test. There is a significant difference in the activity of mutants and controls (Wilcoxon-Mann-Whitney test, P<0.005).

°Pups tested are issued from seven litters, between 4 to 8 hours old. Majority of wild type mice had milk in their stomach but mutant had an empty stomach. There is a significant difference in the activity of mutants and controls (Wilcoxon-Mann-Whitney test, P<0.5e-004).
Table 2: Neuropeptide contents in hypothalamus and pituitary gland of *Magel2* mutant and wild type newborns.

<table>
<thead>
<tr>
<th></th>
<th>Hypothalamus P0 (ng/hypothalamus)</th>
<th>Pituitary Gland P0 (ng/pituitary)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT(n=14) M (Q1,Q3)</td>
<td>KO(n=14) M(Q1,Q3)</td>
<td>P value</td>
<td>WT(n=11) M (Q1,Q3)</td>
</tr>
<tr>
<td><strong>OT</strong></td>
<td>4.2 (3.5,5.7)</td>
<td>2.7 (2.2,3.1)</td>
<td>P&lt;0.005</td>
<td>2.1 (1.9, 2.2)</td>
</tr>
<tr>
<td><strong>AVP</strong></td>
<td>8.4 (8.0,9.2)</td>
<td>6.8 (6.4,7.2)</td>
<td>P&lt;0.001</td>
<td>6.4 (5.8, 7.0)</td>
</tr>
<tr>
<td><strong>OX A</strong></td>
<td>10.1(9.6,11.2)</td>
<td>7.9 (7.5,9.2)</td>
<td>P&lt;0.001</td>
<td>N.D</td>
</tr>
<tr>
<td><strong>ACTH</strong></td>
<td>8.6 (8.5,9.0)</td>
<td>9.1 (8.0,9.4)</td>
<td>N.S.</td>
<td>9.5(8.8,12.2)</td>
</tr>
<tr>
<td><strong>AGRP</strong></td>
<td>4.8 (3.6,6)</td>
<td>5.5 (4.4,7.2)</td>
<td>P&lt;0.05</td>
<td>N.D</td>
</tr>
<tr>
<td><strong>α-MSH</strong></td>
<td>5 (3.7,6.1)</td>
<td>5.6 (5.0,6.5)</td>
<td>N.S.</td>
<td>N.D</td>
</tr>
</tbody>
</table>

Results from Elisa tests in order to compare, between mutant and wild type newborns (at P0), the quantity of several neuropeptides produce in hypothalamus or pituitary gland. Values are indicated as: Median(Quartile1, Quartile3).
Figure 1

a

b

12.8 kb

4.5 kb

376 bp

Necdin

WT

Magel2 KO

Necdin Magel2

Magel2

Necdin

mig

h1.4

z2

N
Figure 2

(a) PS38  VA10  PS38 VA10

(b) PS41  VA4  PS41 VA4

WT  Mega2 KO