Neonatal oxytocin impacts hippocampal network development and restores adult social memory in a mouse model of autism.

Alessandra Bertoni¹, Fabienne Schaller¹, Roman Tyzio¹, Stephane Gaillard², Francesca Santini³, Marion Xolin¹, Diabé Diabira¹, Radhika Vaidyanathan³, Valery Matarazzo¹, Igor Medina¹, Elizabeth Hammock³, Jinwei Zhang⁵, Bice Chini⁴, Jean-Luc Gaiarsa¹, Françoise Muscatelli¹#

¹ Institute of Neurobiology of Méditerranée (INMED), Institut National de la Santé et de la Recherche Médicale (INSERM) UMR 1249, Aix- Marseille Université, Marseille, France.

² Phenotype-expertise, Marseille, France.

³ Florida State University, Tallahassee, FL, USA.

⁴ Institute of Neuroscience, National Research Council (CNR), Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano Milan, and NeuroMI Milan Center for Neuroscience, University of Milano-Bicocca, Italy.

⁵ Institute of Biomedical and Clinical Sciences, College of Medicine and Health, University of Exeter, Hatherly Laboratories, Exeter, EX4 4PS, UK.

#: corresponding author

Email: francoise.muscatelli@inserm.fr.

Institut de Neurobiologie de la Méditerranée (INMED)
INSERM-Aix Marseille Université
Campus Scientifique de Luminy, 13273 Marseille, France

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This study reveals how peripheral administration of oxytocin in newborns treats alterations in the brain social circuits described in a mouse model of autism.

Abstract

Prader-Willi (PW) and Schaaf-Yang (SY) syndromes are genetic neurodevelopmental disorders involving MAGEL2 gene. Magel2-deficient mice mimic the symptoms common to both diseases, in particular autistic-like symptoms. Importantly, peripheral administration of oxytocin during infancy cures social deficiency of Magel2-KO mice beyond treatment into adulthood. However, neurobiological alterations related to oxytocin-signaling and responsible for social deficits are poorly explored in mouse models of autism, including Magel2-deficient mice. Moreover, the mechanisms by which neonatal oxytocin-administration improves social behavior remain unknown. Here, by studying Magel2-KO mice, we aim to decipher the mechanisms underlying the PWS/SYS social alterations and their rescue by oxytocin.

Hippocampal neurons in Dentate Gyrus and CA2/CA3 regions are associated with social memory engrams involving the oxytocin-system. We have shown that Magel2 and oxytocin-receptor are specifically co-expressed in those neurons during development. Then, in Magel2-deficient adult mice, we showed a deficit of social memory and revealed an increase of spontaneous inhibitory activity of pyramidal neurons, a higher number of somatostatin-positive interneurons and an increase in the number of oxytocin-receptors. We also showed a delay in the GABAergic developmental sequence in CA3 neurons associated with biochemical changes in potassium-chloride cotransporter KCC2. Importantly, we demonstrated a strong impact of neonatal oxytocin administration, rescuing all these neuronal alterations.

This study elucidates the mechanisms by which peripheral oxytocin-administration in neonates affects the brain social circuitry. While clinical trials are ongoing, we are demonstrating the therapeutic value of administrating oxytocin in newborns to treat patients with Prader-Willi and Schaaf-Yang syndromes and possibly other neurodevelopmental disorders related to autism.
INTRODUCTION

*MAGEL2* is an imprinted gene that is involved in Prader-Willi (1) and Schaaf-Yang syndromes (2) (PWS and SYS, respectively). Both of these genetic neurodevelopmental disorders present feeding difficulties and hypotonia in infancy but also autistic features with alterations in social behavior and deficits in cognition that persist over the lifespan (3). *Magel2* KO mouse models are pertinent models for both syndromes (3), mimicking poor sucking activity (4), and alterations to social behavior and learning abilities in adulthood (5, 6). *Magel2* mRNA is expressed in the developing hypothalamus until adulthood and *Magel2<sup>tm1.1Mus</sup>* KO neonates display a deficiency of several hypothalamic neuropeptides, particularly OT (4). Daily administration of OT in neonates during the first week of life restores normal sucking activity of *Magel2* KO mice and improves social behavior and learning abilities beyond treatment into adulthood (5). Comparable long term effects have also been reported in other genetic rodent models of autism spectrum disorder (ASD), such as the VPA-induced rat model (7), the Cntnap2 and Fmr1 KO mice (8, 9), and maternal separation (10).

However, the neurobiological alterations involving the OT-system and responsible for social behavior deficits in these models are not known. And, the mechanisms by which OT-treatment in infancy exerts its long-lasting beneficial effects, remain mysterious. At adulthood, OT is thought to regulate aspects of social recognition, social novelty and social memory via interactions with OXTRs in a number of key brain regions (11). With regard to social memory, a critical role has been ascribed to hippocampal OXTR expression in the anterior dentate gyrus (aDG) hilar and anterior CA2/CA3distal regions (aCA2/CA3d) (12-15). In the aCA2/CA3d region, OXTRs are expressed in glutamatergic pyramidal neurons and in GABAergic interneurons, which account for over 90% of OXTR positive cells in the hippocampus (16). Notably, both types of neuron are necessary for the formation of stronger synapses that mediate long term potentiation and social memory (12, 13). During the postnatal development, the mechanisms by which OT-system structures various behaviors is poorly studied. OT-dependent systems may be particularly vulnerable around the neonatal period where OXTR expression is dynamic, with a strong expression in the first two postnatal weeks followed by a decreased expression thereafter (17, 18). Throughout development OT acts on interneurons on the GABAergic activity and also on glutamatergic neurons (19) driving dendritic and synaptic refinement in immature hippocampal glutamatergic neurons (20). Seven clinical trials (phase 1 or 2) using different timing, lasting and dose of OT administration in adults or adolescent with PWS have been conducted and positive or no effects have been reported but no adverse effects. Based on our previous preclinical studies, a phase 2 clinical trial with OT-treatment of infants with PWS significantly improves early feeding and "social
skills” (21), confirming the translational relevance of our study. However, each of the studies suffered important limitations that influences the findings. More research is needed to use properly OT as treatment for PWS or ASD, still we do not yet understand clearly how OT works. We hypothesize that the administration of OT in infancy during a critical developmental stage might be the most beneficial treatment. To validate this hypothesis and propose the best treatment with a strong scientific rationale, it is necessary to elucidate the mechanisms underlying the cause and the long-lasting rescue of PWS/SYS alterations by OT. Here, we aimed to clarify the physiological and cellular mechanisms related to the OT-system that are disturbed in Magel2tm1.1Mus-deficient mice and those responsible for the long-term rescue effects following OT peripheral administration in pups. We focused our study on the deficit of social memory, a robust phenotype linked to OT-system.

RESULTS

To overcome the phenotypic heterogeneity of the heterozygous +m/-p Magel2tm1.1Mus mouse, due to the stochastic expression of the maternal Magel2 allele when the paternal allele is deleted (22), Magel2tm1.1Mus homozygous (-/-) mice were used (“Magel2 KO” hereafter). In the following experiments WT and Magel2 KO pups were naïve or treated for four days (Postnatal day P0, P2, P4 and P6) with one subcutaneous administration of physiological saline (“vehicle”) or oxytocin (2 µg “OT-treatment” or “+OT") per day.

Deficits of social memory in Magel2 KO adults are rescued by neonatal OT-treatment.

At adulthood, we focused on social behavior using the three-chamber test in order to assess social exploration (sociability), the preference for social novelty (social discrimination) and social memory (short-term social memory) (23) (Figure 1A). Magel2 KO males showed levels of sociability and social discrimination similar to WT males but exhibited a significant deficit in social memory (Figure 1B, Figure 1-figure supplement 1). As previously reported (24), we observed a failure of the three-chamber test in revealing sociability in the cohort of female mice (Figure 1-figure supplement 2); a result reproduced in second cohort of WT females (data not shown). As a consequence, we restricted all following studies to male mice.

First, the effects of neonatal vehicle and OT-treatment were assessed in WT male pups at adulthood in the three-chamber test. We found that neither treatment had any measurable effect on sociability, social discrimination or social memory: the amount of time spent sniffing in different compartments was similar to that recorded in untreated WT males (Figures 1 B-C). Furthermore, no significant effects of neonatal OT-treatment were detected on adult performance of novel object recognition (Figure1-Supplement 3A), open field (Figure 1-Supplement 3B) or elevated plus maze tests (Figure1-Supplement 3C). Thus, neonatal OT-
treatment has no significant adverse or beneficial long-lasting effects on widely used assays of social behavior, object recognition, anxiety-like or motor behaviors in WT animals but a reduction in the distance moved in the open-field in females.

Unsurprisingly, Magel2 KO-vehicle males presented a social memory deficit similar to untreated Magel2 KO males (Figure 1D-B). However, this was rescued by neonatal OT-treatment (Figure 1D). Sociability and social discrimination indices were not affected by Magel2 deletion or OT-treatment (Figure 1-Supplement 1).

Thus, the loss of Magel2 causes a deficit in social memory in male Magel2 KO adults. This deficit was rescued by a neonatal OT-treatment. Due to the robust effect observed on social memory, we focused our subsequent investigations on the hippocampal region, previously shown to be specifically involved in OT-mediated effects on social memory (12, 13).

**aDG and aCA2/CA3d regions are activated by the social memory test in WT and Magel2 KO mice.**

Cells in the aCA2/CA3d and aDG regions have been previously shown to express OXTRs and are involved in social memory (12, 13). We therefore asked whether these cells could be activated by the social memory test. WT and Magel2 KO mice were sacrificed 90 min after the end of social memory test (+SI, for Social Interactions) and their brains examined for cFos immunolabeling, a marker of neuronal activity (Figure 2A). We found a similar quantity of cFos positive cells in untested WT (WT-SI) and Magel2 KO (Magel2 KO-SI) mice and a significant increase (83%) in the number of cFos+ cells in the *stratum pyramidale* of aCA2/CA3d in tested WT (WT+SI) compared with WT-SI (Figure 2D); in Magel2 KO+SI mice we observed a 25% significant increase of cFos+ cells compared with WT+SI (Figure 2E). In the aDG, mainly in the hilus and *stratum granulare*, a significant increase of ~60% of cFos+ cells was observed in both WT+SI and Magel2 KO+SI compared with WT or Magel2 KO-SI. Overall, these data confirm a strong activation of neurons in aDG and aCA2/CA3d regions following social memory test in both genotypes (WT and Magel2 mice) with a 25% increase of cFos activated cells observed in the aCA2/CA3d region in Magel2 KO+SI compared with WT+SI.

**Magel2 and Oxtr are both co-expressed in the aDG and aCA2/CA3d regions**

Magel2 is known to be highly expressed in hypothalamus in particular in the PVN, while its expression in hippocampal regions is less well characterized. In adults (P56, Allen Brain Atlas) and in juveniles (P28) using RNAscope technique (Figure 3A), we could confirm expression of Magel2 transcript in the CA3 deep layer of the *stratum pyramidale* and in the DG with a co-expression with Oxtr mRNA in some cells. Then, to define more precisely the cell types expressing Magel2 and Oxtr, we consulted two public RNAseq data libraries and we extracted data from cortex and hippocampal samples (Allen brain; Linnarson lab: http://celltypes.brain-
map.org/rnaseq/mouse/cortex-and-hippocampus; http://mousebrain.org/genesearch.html). It appears that \textit{Oxtr} and \textit{Magel2} are co-expressed in the CA3 excitatory neurons (expressing CCK) of adult mice and also in several interneuron sub-populations expressing SST or SST and PV and in the axo-axonic long-range projections interneurons and the basket bistratified cells. These sub-populations of interneurons represent a small number of cells that have a wide and diverse spectrum of actions. Indeed, we confirmed the co-expression of \textit{Oxtr} and \textit{Sst} mRNAs in hippocampus (Figure 3B).

Taking in account the dynamic expression of OXTRs, we looked at the expression of \textit{Magel2} and \textit{Oxtr} transcripts in the anterior hippocampus in infancy, at P7 (Figure 3A). At P7, we detected \textit{Oxtr} and \textit{Magel2} mRNAs in the aCA2/CA3d region with \textit{Magel2} more expressed in the deep layer of the \textit{stratum pyramidale}. At P28, the level of \textit{Magel2} transcripts was reduced but still present in the deep layer of aCA2/CA3d region and \textit{Oxtr} transcripts were also strongly expressed in pyramidal cells. Expression of \textit{Magel2} and \textit{Oxtr} was also detected in few cells of the \textit{stratum oriens} and \textit{stratum radiatum} where co-expression can be observed. In the DG, an expression of \textit{Oxtr} and \textit{Magel2} is detected in the hilus, some cells presenting a clear co-localization of both transcripts.

A deficit of mature oxytocin produced by hypothalamus was previously revealed in \textit{Magel2} KO pups (4). In addition, these data suggest that the lack of \textit{Magel2} can also alter the development and function of \textit{Oxtr} expressing excitatory neurons in the aCA2/CA3d region and also of the SST or PV interneurons expressing \textit{Oxtr}.

**The quantity of OT-binding sites is increased in the aCA2/CA3d and aDG regions of \textit{Magel2} KO adult mice and normalized in the aDG following an OT-treatment**

We then looked at the distribution of OT-binding sites in \textit{Magel2} KO-vehicle or \textit{Magel2} KO+OT compared with WT-vehicle hippocampi by autoradiography (Figure 4). We observed a significant increase of OT-binding sites in the aCA2/CA3 (100%, Figure 4A-B) and aDG (80%, Figure 4C-D) regions but not in the ventral CA1/CA2/CA3 region (Figure 4E-F). In \textit{Magel2} KO+OT we observed a normalization of the quantity of OT-binding sites in the aDG, but not in the aCA2/CA3 region where OT-binding sites level was decreased but remained elevated compared to WT (Figure 4A-B). The binding study indicate subregions specific modulation of OXTR in the hippocampus. We then asked if this subregion specific effect could be linked to specific changes in neuronal subpopulations in the same regions.

**The number of SST+ neurons is increased in the aCA2/CA3d and aDG regions of \textit{Magel2} KO adult mice and normalized following an OT-treatment.**
In the anterior adult hippocampus OXTRs are expressed mainly in pyramidal cells of aCA2/CA3d region and mainly in SST and/or PV interneurons of aDG (see above, (12, 16)). So we quantified the number of SST+ and PV+ cells in Magel2KO versus WT adult hippocampi and observed a significant higher number of SST+ cells in both aCA2/CA3d (1.6 x) and aDG (1.8 x) regions of Magel2 KO animals (Figure 5 A-F and M-N). In contrast, the number of SST+ cells was slightly but significantly decreased in both aCA2/CA3d (less 12%) and DG regions (less 17%) in OT-treated Magel2 KO mice compared with untreated WT mice (Figure 5 G-L and O-P). An increase of SST+ neurons was observed in anterior hippocampus of adult Magel2 KO and, conversely, OT-treatment of Magel2 KO pups was associated with a slight decrease of SST+ neurons in adult mutant compared with WT hippocampi. PV+ cells were equally abundant in both genotypes (Figure 5-figure supplement 1). This change in the quantity of SST+ cells might have consequences in the alteration of the excitation/inhibition (E/I) balance.

GABAergic and glutamatergic activities are altered in Magel2 KO aCA3d pyramidal neurons and neonatal OT-treatment normalizes the GABAergic activity but reduces the glutamatergic activity in WT mice.

Alterations in GABA/glutamate (Excitatory/Inhibitory; E/I) balance is an electrophysiological feature frequently associated with multiple neurodevelopmental disorders. In addition, here, with an alteration of the quantity of OXTRs and of SST+ cells, we expect an alteration of the E/I ratio. Hippocampal brain slices of WT, Magel2 KO, WT+OT and Magel2 KO+OT male mice (P25-P30) were analyzed using whole cell patch clamp to record the activity of aCA3d pyramidal neurons (Figure 6A). Spontaneous activities analysis (Figure 6B) revealed a reduced amplitude of postsynaptic glutamatergic currents (sGlut-PSCs) in Magel2 KO as compared to WT mice (x1.7 less, Figure 6D), while the frequency of sGlut-PSCs was not changed (Figure 6C). The same Magel2 KO neurons presented a significant increase in GABAergic (sGABA-PSCs) frequency (x1.8, Figure 6E) while the amplitude of sGABA-PSCs was similar to that of WT (Figure 6F). Patch clamp recordings of the glutamatergic and GABAergic miniature currents (mGlut-PSCs and mGABA-PSCs, respectively) showed a significant reduction in amplitude of mGlut-PSCs, with no change in their frequency, in Magel2 KO neurons compared to WT (x1.4 less, Figure6-figure supplement1B-C), but no differences in frequencies and amplitudes of mGABA-PSCs (Figure6-figure supplement1D-E).

An abnormal dendritic morphology of the Magel2 KO CA3 pyramidal neurons could be associated with the alterations of the GABAergic or glutamatergic activities. We defined the morphology of recorded aCA3d neurons by adding biocytin in the recording solution and performing Neurolucida reconstruction followed by a Sholl analysis. No differences were
revealed in dendritic morphology between Magel2 KO and WT CA3 pyramidal neurons in juvenile mice (Figure 6-figure supplement 2).

Altogether, those results show that, in Magel2 KO pyramidal neurons of aCA3d, there is a significant increase in the GABA/Glutamate ratio with no change in their neuronal morphology.

We next investigated the effects of OT-treatment on the GABA/Glutamate balance in WT and Magel2 KO. Quite unexpectedly, the spontaneous glutamatergic activity was significantly reduced in WT+OT mice compared with WT: frequency was considerably reduced (x 2.7 less), being even lower than in OT-treated mutants (Figure 6C) and the amplitude was reduced (x1.5 less), being similar to the one observed in mutant mice (Figure 6D). These findings indicate that OT-treatment reduced strongly the glutamatergic activity in WT mice without any apparent impact on the behavioral outcome measured (see above). In Magel2 KO, OT-treatment in the first week of life decreased significantly the frequency of sGABA-PSCs restoring a frequency similar to WT (Figure 6E). There was no effect of OT-treatment on the amplitude of sGABA-PSCs (Figure 6F). However, on the same pyramidal neurons, such treatment in Magel2 KO+OT reduces significantly the frequency of sGlut-PSCs (x2 less) compared with the frequency recorded in WT or in the Magel2 KO mice (Figure 6C). The amplitude of sGlut-PSCs was not changed in Magel2 KO+OT compared with Magel2 KO, being reduced in the untreated or OT-treated mutants compared with WT mice (Figure 6D). These results show that OT administration in the first week of life normalized the frequency of spontaneous GABAergic activity and reduced significantly the frequency of spontaneous glutamatergic activity in Magel2 KO compared with WT untreated mice. OT-treatment in WT juvenile mice reduced strongly the glutamatergic activity (frequency and amplitude).

The excitatory-to-inhibitory developmental GABA-shift is delayed in Magel2 KO hippocampal neurons

Because Oxtr and Magel2 are co-expressed in aCA2/CA3d hippocampus in infancy (see above at P7), and because in Oxtr KO mice (25) as in several models (26) of autism the depolarizing to hyperpolarizing (D-to-H) developmental GABA shift is delayed, we investigated the GABA shift timing in Magel2 KO pups. First, we performed calcium (Ca\textsuperscript{2+}) imaging experiments by measuring the percentage of neurons showing GABA-induced Ca\textsuperscript{2+} responses in developing hippocampal neuronal cultures of Magel2 KO and WT embryos collected at E18.5 (DIV0 for days in vitro 0) (Figure 7A-B). At DIV4, we found a significant two-fold higher proportion of Magel2 KO neurons increasing Ca\textsuperscript{2+} upon GABA stimulation compared with WT. At DIV8 and DIV11, the percentage of responsive neurons was markedly decreased and similar in both genotypes. We checked that the amplitude of Ca\textsuperscript{2+} responses were similar in both genotypes (Figure 7-figure supplement 1A). Noticeably, comparing the KCl-induced Ca\textsuperscript{2+} responses between WT and Magel2 KO, we found no increase and even a significant reduction
at DIV2, DIV4 and DIV8 in Magel2 KO cultures (Figure 7-figure supplement 1B), suggesting that the voltage-operated calcium channels were not responsible for the significant higher proportion of Magel2 KO neurons increasing Ca$^{2+}$ upon GABA stimulation. Altogether, these results showed a developmental delay in GABA-induced Ca$^{2+}$ responses in cultures of Magel2 KO embryonic hippocampal neurons and suggest a developmental GABA-shift delay in Magel2 KO hippocampal neurons.

To further examine whether the action of GABA was altered in Magel2 KO hippocampal neurons, cell-attached recordings of single GABA$_A$ channels/receptors were performed in acute brain slices in order to measure the Driving Force of GABA$_A$ (DF$_{GABA}$). DF$_{GABA}$ translates the differences in the actions of GABAergic synapses via GABA$_A$ receptors revealing a hyperpolarizing or depolarizing response of GABA, associated with mature or immature neurons, respectively. Thus, we measured the DF$_{GABA}$ in aCA3 pyramidal cells (PCs) at postnatal days 1 (P1), 7 (P7) and 15 (P15), on acute brain slices obtained from male mice (Figure 7C). At P1, both Magel2 KO and WT mice did not show significant differences in the DF$_{GABA}$ but a tendency to an increase of DF$_{GABA}$ in Magel2 KO compared with WT. Significant difference in the DF$_{GABA}$ was observed at P7 with a 1.8-fold increase of the DF$_{GABA}$ value in mutant neurons, whereas a similar DF$_{GABA}$ was obtained at P15. Since Magel2 and Oxtr are expressed in interneurons (INs), in which a GABA shift has been also described (27), we also measured the DF$_{GABA}$ in INs and observed similar values in CA3 interneurons of mutant and WT mice (Figure 7-figure supplement 1C). Noticeably, at P7, the resting membrane potential (Figure 7-figure supplement 1D), the conductance (Figure 7-figure supplement 1F) and the capacitance (Figure 7-figure supplement 1E) did not differ statistically between WT and Magel2 neurons. Altogether these data suggest a transient higher GABA depolarizing activity at P7 in CA3 pyramidal neurons of Magel2 KO pups and consequently a delay in GABA-shift.

Then, at P7, we assessed the effect of the in vivo OT-treatment in Magel2 KO and WT animals compared with WT-vehicle pups (Figure 7D). Both Magel2 KO+OT and WT+OT mice showed a significant decrease in the DF$_{GABA}$ values compared with WT-vehicle, suggesting a reduction in GABA depolarizing activity following an OT administration in infancy.

**Post-translational changes in cation-chloride co-transporter KCC2 in Magel2 KO hippocampus**

We then asked if the altered DF$_{GABA}$ values could be due to alteration in the expression of the neuronal transporters of Cl$^-$ in Magel2 KO mice. The neuronal level of [Cl$^-$] and Cl$^-$ dependent depolarizing or hyperpolarizing strength of GABA are determined by complex mechanism involving primarily Cl$^-$ extrusion by potassium/chloride cotransporter type 2 (KCC2) whose expression increases progressively during neuronal maturation (28). In developing WT
hippocampal neurons, the emerging activity of KCC2 contributes to progressive lowering of $[\text{Cl}^-]$ that at P7 shifts GABA action from depolarizing to hyperpolarizing. As consequence, the activation of GABA$_A$R produces neuronal Cl$^-$ influx. The quantitative western blot analysis of the total KCC2 protein expression in hippocampi of P7 mice did not reveal statistically significant difference of the amount of KCC2 between WT and Magel2 KO animals (Figure 8A-B). The ion-transport activity of KCC2 and its stability at the cellular plasma membrane also strongly depend on post-translational modifications of multiple phosphorylation sites (29). We therefore applied in a next step phospho-site-specific antibodies, as they were previously shown to quantitatively monitor changes in KCC2 phosphorylation (30-32). Currently, a limited number of such phospho-specific antibody is available. They are directed against the well-known KCC2's phospho-sites Ser$^{940}$ (33) and Thr$^{1007}$ (31, 32). Western blot analysis revealed that the Magel2 KO hippocampi (as compared to WT) were characterized by significantly decreased amount of the phosphorylated form of Ser$^{940}$ (P-Ser$^{940}$). The amount of phosphorylated Thr$^{1007}$ (P-Thr$^{1007}$) was not statistically different, but albeit higher in Magel2 KO mice (Figure 8A-B). Thus, in Magel2 KO mice there was significant decrease of P-Ser$^{940}$ (inactivation of function (33)) and no change of P-Thr$^{1007}$ whose progressive developmental de-phosphorylation is associated with increase of KCC2 activity (31). At P7, the decreased P-Ser$^{940}$/P-Thr$^{1007}$ ratio in Magel2 KO mice may thus result in predominance of KCC2 internalization over surface expression. As a consequence of the decreased amount of surface expressed molecules, the Cl$^-$ extrusion ability of KCC2 is decreased, causing an increase of $[\text{Cl}^-]$, and could induce a depolarizing shift of GABA described above (Figure 8C).

DISCUSSION

Here we showed that Magel2 KO mice display a social memory deficit at adulthood, that is a stereotypical phenotype of several models characterized by a deficit in OT-system (34). Furthermore, hippocampal neurons in Dentate Gyrus and CA2/CA3 regions are associated with social memory engrams involving the OXTR-expressing neurons and Magel2 is expressed in these neurons. In Magel2 KO as in WT mice, we observed a specific neuronal activity in both regions following the social memory test; this activity is more increased in the CA2/CA3 Magel2-deficient hippocampi compared with WT. Then, we unraveled several changes. Magel2 deficiency alters the GABAergic developmental sequence probably resulting from post-translational biochemical modifications of KCC2. At adulthood, we have also revealed an increase in the quantity of OT-binding sites, a higher number of SST-positive interneurons and an increase in the GABAergic activity of pyramidal neurons. Interestingly a peripheral administration of OT in the first days after birth rescues all those changes in Magel2 KO mice.
The effect of peripheral OT-administration in Magel2 KO neonates

In our previous study and here, we have shown long-term and beneficial effects of a peripheral administration of OT in Magel2 KO neonates that rescues nearly all social and cognition deficits described in adult Magel2 KO mice (5). However, in our previous study we did not investigate neither the neurobiological causes of these deficits nor the effects of OT on those alterations. We focused this study on social memory because the mechanisms by which OT controls social memory via the OXTR-expressing neurons in hippocampus are described. We clearly demonstrated that the neurobiological alterations found in Magel2 KO mice involve the brain OT-circuitry, at least in hippocampus, and peripheral administration of OT in neonates impacts the OT-targeted neurons. Thus, those results give a clear and positive response to the debated question on whether or not a peripheral administration of OT in neonates acts on the brain. Is it a direct or indirect effect? It might be both. Today, several studies converge to propose that peripheral OT goes through the Brain Blood Barrier via an active mechanism (RAGE transporters) and/or a passive mechanism in neonates when the BBB is more permeable. The observed long-lasting OT effects could result from a strong impact of OT administration in key developmental hippocampal processes such as the developmental GABA-shift (as discussed below) and can also be achieved by epigenetic modifications that impact gene expression such as the Oxtr expression, as observed in prairie voles following a maternal OT administration (35). Transcriptomic and proteomic studies at different developmental ages would help to understand the life-long effect of an early OT-treatment in mutant and WT mice.

The lack of Magel2 alters the OT-system: causes and consequences

Previously we have described a deficit in the quantity of the released mature form of OT in the brains of neonates and we detected an accumulation of the intermediate non-mature forms of OT suggesting a problem of processing (4). Noticeably, Ates et al. (36) showed, ex vivo, that lack of Magel2 expression is associated with significant suppression of the overall activity of oxytocin neurons. Furthermore, here we showed a co-expression of Magel2 and Oxtr transcripts in the same neurons. Thus, given the role of MAGEL2 in ubiquitination, actin regulation and endosomal sorting processes (37), the absence of Magel2 expression could induce a wide range of post-translational modifications of various molecular and cellular processes in OT and OXTR expressing neurons. Altogether those data suggest that dysregulation of the OT-system in Magel2 KO mice goes beyond OT expression.

In agreement with the role of Magel2 in OXTR-expressing neurons, here we showed that the excitatory-to-inhibitory GABAergic developmental sequence is transiently delayed in Magel2 KO mice during the first week of life, with GABA\(_A\)-mediated responses more depolarizing in Magel2 KO that WT pyramidal neurons at P7. We further showed that this electrophysiological deficit corresponds well with decreased functional KCC2 at the cell membrane, caused by a
deficit of KCC2 phosphorylation (on Ser\textsuperscript{940}). Notably, Ser\textsuperscript{940}phosphorylation is controlled by OXTR activation via a PKC-dependent pathway and allows translocation of KCC2 to the cell membrane (25), enhancing KCC2 mediated Cl\textsuperscript{−} transport (33, 38). This mechanism is relevant to control the GABAergic developmental sequence \textit{in vitro} and possibly \textit{in vivo} (39). The functional consequences of this delayed developmental GABA-shift in \textit{Magel2} KO pyramidal neurons are not clearly established. However, there are compelling reasons to suspect that transient disruption of GABAergic maturation in the immediate postnatal period could be enough to permanently alter neural circuit dynamics. Indeed, P7 is a critical milestone in the development of GABAergic neurons in the mouse neocortex and hippocampus, characterized by major changes in network dynamics (e.g. end of \textit{in vitro} giant-depolarizing-potentials (40) and \textit{in vivo} early sharp waves (41)), intrinsic membrane properties (e.g. input resistance) and synaptic connectivity (42). Altogether those data suggest that the absence of \textit{Magel2} delays neuronal maturation during this critically vulnerable period of brain development, resulting in a distinct adult phenotype. Whether the delay of the GABA-shift alone is sufficient to derail neurotypical developmental trajectory remains a key question for future study: notably, similar or longer GABA-shift delays have been observed in several models of autism (43-45) and in \textit{Oxtr} KO mouse models (25). Recently, Kang et al. (46) showed in \textit{Disc1} KO mouse model, that elevated depolarizing GABA signaling is a precursor for the later E/I imbalance (in favor of inhibition) and social impairment. Similarly, we showed that, in a KCC2 mutant mouse, the GABA shift delay is responsible for the E/I alteration (39). Importantly, OT-treatment has an opposite action on the excitatory-to-inhibitory GABA shift with a relative hyperpolarizing effect at P7 in \textit{Magel2} KO and WT pups compared with WT-vehicle animals. This effect of OT-treatment might modify the maturation of the hippocampal circuitry.

**The E/I ratio and social behavior**

Reductions in synaptic signal-to-noise ratio in cortical and hippocampal pyramidal neurons, driven by a change in the ratio of dendritic excitatory and inhibitory synapses, are widely thought to contribute to reduced efficiency of signal processing in ASD, a mechanism known as the E/I ratio hypothesis (47). We confirm E/I imbalance characterized by increased GABAergic activity and lower glutamatergic activity in CA3 neurons in \textit{Magel2} KO mice, consistent with observations made in other ASD models (48-51). Furthermore, we report that perinatal OT administration restored normal GABAergic activity in \textit{Magel2} KO mice without improving glutamatergic transmission. Unexpectedly, perinatal OT treatment has a significant impact on the WT neurons inducing a strong reduction of glutamatergic activity without affecting GABAergic activity. This is a significant observation, because it shows that, although the ASD-like behavior \textit{Magel2} KO animals is correlated with a change in E/I ratio, E/I imbalance
in OT treated WT animals was not sufficient to drive detectable changes in social behavior or cognitive performance. We therefore conclude that E/I imbalance characterized by isolated decreased spontaneous glutamatergic transmission is unlikely to underlie the ASD traits investigated here, and suggest that an upper threshold of GABAergic or glutamatergic activity, but not the E/I ratio per se, may be important for normal development.

**Role of oxytocin receptors and somatostatin neurons**

In adult *Magel2* KO mice we observed increased OT-binding in the DG and CA2/CA3 regions of the anterior hippocampus compared to WT mice. OT administration in *Magel2* KO neonates normalized hippocampal OT binding sites in adulthood, suggesting that the increased expression of OXTR observed in *Magel2* KO hippocampus may be a consequence of the reduced OT production reported in these animals (4). This observation supports the idea that life-long OXTR expression is to some extent determined by early life OT binding, described as a “hormonal imprinting” effect (35, 52).

Since DG and CA2/CA3 hippocampal OXTRs are expressed in PV and SST interneurons, we quantified those populations and found a significant increase in the number of aDG and aCA2/CA3 SST+ neurons in mutant mice, while the number of PV+-interneurons was not modified. OT-treatment normalized the number of SST-expressing neurons in *Magel2* KO pups, revealing a causal link between the administration of OT in infancy and the quantity of SST+-neurons. This result may reflect actual changes in the number of SST+-neurons, or alternatively changes in SST expression and hence more reliable detection of SST-synthesizing neurons. Interestingly, OT modulates the activity of the SST+-neurons, increasing the excitability of SST interneurons (53) but no studies report an effect of OT on SST production.

SST interneurons have recently been shown to play a role in the modulation of social behavior (54, 55) and a link between altered social memory and an increase in SST cell number has been recently suggested in LPS-treated female neonates (56). It is tempting to speculate that OXTR-transmission regulates the activity of SST hippocampal interneurons and the production/release of mature SST and impacts social memory. Further work is needed to fully characterize the role of OXTRs on SST interneurons in relation with social memory.

**Conclusions**

The mechanisms by which peripheral OT administration improve social behavior in *Magel2*-deficient mice or in other models of autism remained unexplored. Here, in *Magel2* KO mice, we have clarified the oxytocin-dependent physiological and cellular mechanisms in the hippocampus underlying the loss of social memory. An open question is whether developmental alterations in the *Magel2* KO are a consequence of dysfunction in a
single sequential pathway (depending on the developmental GABA shift) or whether they reflect parallel interconnected circuits in the developing hippocampus. Surprisingly, peripheral OT-treatment in neonates permanently rescued nearly all functional and cellular hippocampal alterations that were identified in Magel2 KO mice. A significant impact of OT-treatment was also observed in WT animals but all performed behavioral tests were normal. Overall, we have shown that OT-treatment in infancy has a significant impact and rescues permanent specific hippocampal alterations in Magel2 KO mice at different developmental ages, many of these hippocampal alterations have been described in several models of neurodevelopmental disorders with ASD (see above). In addition, OT deficit has often been described in rodent models of ASD (see introduction). Taken together, our findings reinforce the idea that OT-treatment in early life may represent a viable therapeutic strategy for patients with SYS or PWS and possibly other neurodevelopmental disorders.

MATERIALS AND METHODS

Animals

To perform functional studies, we chose to work with the Magel2\textsuperscript{tm1.1Mus} homozygous (-/-) mice, here named Magel2 KO, in order to have a greater homogeneity in the values, allowing a better analysis of the effects of the mutation. Magel2\textsuperscript{tm1.1Mus+/-} (WT) and Magel2\textsuperscript{tm1.1Mus-/-} (Magel2 KO) mice were maintained on a C57BL/6J genetic background and stabulated in standard conditions, with \textit{ad-libitum} access to food and water. Mice were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (N.R.C., 1996) and the European Communities Council Directive of September 22th 2010 (2010/63/EU, 74). Experimental protocols were approved by the institutional Ethical Committee guidelines for animal research with the accreditation no. B13-055-19 from the French Ministry of Agriculture. We maintain grouped-house mice (3-5 mice/cage). All efforts were made to minimize the number of animals used. Magel2-deficient mice were generated as previously described (4). Due to the parental imprinting of Magel2 (paternally expressed only), to obtain heterozygote mice (+m/-p), males carrying the mutation on the maternal allele (-m+/p) were crossed with wild-type C57BL/6J females. To obtain homozygote mice, Magel2 KO homozygote males and females were crossed. Importantly, we checked that Magel2 KO mothers had a similar maternal behavior as WT mothers. All mice were genotyped by PCR starting from DNA extracted from tail snips (around 3 mm), using the following couples of primers: MI2KO F (5’-CCCTGGGTTGACTGACTCAT-3’) and MI2KO R (5’-TCTTCTTCCTGTGGGCTTTG-3’) to discriminate the mutant allele from the WT, 71456 F (5’-CACTCGATCACGTATGGCTCCATCA-3’) and 71457 R (5’-
GATGGCAGGCACCTGACTTACATGCTG-3') to discriminate the heterozygous from the homozygous mice.

**Oxytocin Treatment**

WT pups and Magel2 KO pups were removed from their mother, placed on a heating pad, given a subcutaneous (s.c.) injection and quickly returned to the mother. The solutions injected were isotonic saline (10 µl) for the control mice and 2 µg of OT (Phoenix Pharmaceuticals Inc., cat #051-01) diluted in isotonic saline (10 µl) for the treated mice. The treatment was performed during the first week of life every other day (P0, P2, P4, P6).

**Behavior**

All the behavioral tests were performed by Phenotype Expertise, Inc. (France). For all tests, animals were first acclimated to the behavioral room for 30 minutes.

**Elevated-Plus Maze.** The EPM is used to assess anxiety state of animals. The device consists of a labyrinth of 4 arms 5 cm wide located 80 cm above the ground. Two opposite arms are open (without wall) while the other two arms are closed by side walls. The light intensity was adjusted to 20 Lux on the open arms. Mice were initially placed on the central platform and left free to explore the cross-shaped labyrinth for 5 minutes. Maze was cleaned and wiped with H₂O and with 70% ethanol between each mouse. Animal movement was video-tracked using Ethovision software 11.5 (Noldus). Time spent in open and closed arms, the number of entries in open arms, as well as the distance covered, are directly measured by the software.

**Open-field.** Open-field test was performed in a 40 x 40 cm square arena with an indirect illumination of 60 lux. Mouse movement was video-tracked using Ethovision software 11.5 (Noldus) for 10 minutes. Total distance traveled and time in center (exclusion of a 5 cm border arena) are directly measured by the software. Grooming (time and events) and rearing were manually counted in live using manual functions of the software, by an experimented behaviorist. The open-field arena was cleaned and wiped with H₂O and with 70% ethanol between each mouse.

**New object recognition.** The arena used for the novel object recognition test was the same used for the open-field test. The arena was cleaned and wiped with 70% ethanol between each mouse. Two identical objects (50 ml orange corning tube) were placed in the opposite corners of the arena, 10 cm from the side walls. The tested mouse was placed at the opposite side of the arena and allowed to explore the arena for 10 min. After 1h, one object was randomly replaced with another novel object, which was of similar size but differ in the shape and color with the previous object (white and blue lego bricks). Then, the same mouse was placed in the arena and allowed to explore the two objects (a new and an "old" familiar object) for 10 min.
The movement of the mice was video-tracked with Ethovision 11.5 software. Time of exploration of both objects (nose located in a 2 cm area around object) was automatically measured by the software.

**Three-chamber social preference test.** The test was performed as described previously (23). The three-chamber apparatus consisted of a Plexiglas box (50x25 cm) with removable floor and partitions dividing the box into three chambers with 5-cm openings between chambers. The task was carried out in four trials. The three-chambers apparatus was cleaned and wiped with 70% ethanol between each trial and each mouse.

In the first trial (habituation), a test mouse was placed in the center of the three-chamber unit, where two empty wire cages were placed in the left and right chambers to habituate the test mouse to arena. The mouse was allowed to freely explore each chamber. The mouse was video-tracked for 5 min using Ethovision software. At the end of the trial, the animal was gently directed to the central chamber with doors closed. In the second trial (social exploration), a 8-weeks old C57BL/6J mouse (S1) was placed randomly in one of the two wire cages to avoid a place preference. The second wire cage remained empty (E). Then, doors between chambers were opened and the test mouse was allowed to freely explore the arena for 10 min. At the end of the trial, animal was gently directed to the central chamber with doors closed. A second 8-weeks old C57BL/6J mouse (S2) was placed in the second wire cage for the third trial (social discrimination). Thus, the tested mouse had the choice between a familiar mouse (S1) and a new stranger mouse (S2) for 10 min. At the end of the trial, the mouse was returned to home-cage for 30 min. For the fourth trial (short-term social memory), S2 was replaced by a new stranger mouse (S3), the familiar mouse (S1) staying the same. Then tested mouse was allowed to freely explore the arena for 10 min. Time spent in each chamber and time of contact with each wire cage (with a mouse or empty) were calculated using Ethovision software. The measure of the real social contact is represented by the time spent in nose-to-nose interactions with the unfamiliar or familiar mouse. This test was performed using grouped-house mice of 4 months old.

**Primary hippocampal cultures**

Embryonic day 18 dissociated hippocampal neurons were obtained from wild-type and Magel2 KO timed pregnant mice as previously described (57) with slightly modifications here described. Briefly, the hippocampi of E18 embryos were dissociated by an enzymatic treatment (0.25% trypsin for 18 min at 37°C) followed by mechanic dissociation with a fire-smoothed Pasteur pipette or p1000µl/p200µl tips. For calcium imaging experiments, 200 000 cell/well (in MW 6 wells) were plated on round 26 mm glass coverslips pre-coated with poly-L-lysine containing Neurobasal medium (Life Technologies) augmented with B27 supplement (2% v/v;
Life Technologies), L-glutamine (2mM), penicillin/streptomycin (100U/ml) and 25µM Glutamate. This media was replaced with glutamate-free media after 5 hours. Neurons were then maintained at 37°C in humidified atmosphere (95% air and 5% CO₂), and half of the medium was refreshed twice a week.

**Calcium imaging recordings**

Calcium imaging experiments were carried out as previously described (25). Briefly, hippocampal neurons were loaded with the membrane-permeable fluorescent Ca²⁺ indicator Fura-2/AM (1µM; SigmaAldrich) for 40 min at 37°C, 5% CO₂. The cells were then placed into the recording chamber of an inverted microscope (Axiovert 100, Zeiss), washed with the extracellular recording solution, KRH buffer, and imaged through a 40x objective (Zeiss). Fura-2/AM was excited at 380 nm and at 340 nm through a Polychrom V, (TILL Photonics GmbH) controlled by the TillVisiON software 4.01. Emitted light was acquired at 505nm at 1Hz, and images collected with a CCD Imago-QE camera (TILL Photonics GmbH). The fluorescence ratio F340/380 (ΔF340/380) was used to express Ca²⁺ concentrations in regions of interest (ROI) corresponding to neuronal cell bodies. 100µM GABA was administered in the recording solution and temporal changes in ΔF340/380 were followed. Increases in ΔF340/380 higher than 0.04 units were considered reliable Ca²⁺ responses. After wash with KRH buffer and recover, KCl (50mM) was administered to identify viable neurons. Responses with a ΔF340/380 smaller than 0.1 units were excluded from the analyses. From DIV8 on, 1 µM TTX (Tocris, cat #1069) was added to this extracellular recording solution.

**Hippocampal slice preparation and electrophysiological recordings**

Brains were removed and immersed into ice-cold (2-4°C) artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 126 NaCl, 3.5 KCl, 2 CaCl₂, 1.3 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃ and 11 glucose, pH 7.4 equilibrated with 95% O₂ and 5% CO₂. Hippocampal slices (400 µm thick) were cut with a vibrating microtome (Leica VT 1000s, Germany) in ice cold oxygenated choline-replaced ACSF and were allowed to recover at least 90 min in ACSF at room (25°C) temperature. Slices were then transferred to a submerged recording chamber perfused with oxygenated (95% O₂ and 5% CO₂) ACSF (3 ml/min) at 34°C. **Whole-cell patch clamp recordings** were performed from P20-P25 CA3 pyramidal neurons in voltage-clamp mode using an Axopatch 200B (Axon Instrument, USA). To record the spontaneous and miniature synaptic activity, the glass recording electrodes (4-7 MΩ) were filled with a solution containing (in mM): 100 KGlutonate, 13 KCl, 10 HEPES, 1.1 EGTA, 0.1 CaCl₂, 4 MgATP and 0.3 NaGTP. The pH of the intracellular solution was adjusted to 7.2 and the osmolality to 280 mOsmol l⁻¹. The access resistance ranged between 15 to 30 MΩ. With
this solution, the GABA<sub>A</sub> receptor-mediated postsynaptic current (GABAA-PSCs) reversed at -70mV. GABA-PSCs and glutamate mediated synaptic current (Glut-PSCs) were recorded at a holding potential of -45mV. At this potential GABA-PSC are outwards and Glut-PSCs are inwards. Spontaneous synaptic activity was recorded in control ACSF and miniature synaptic activity was recorded in ACSF supplemented with tetrodotoxin (TTX, 1µM). Spontaneous and miniature GABA-PSCs and Glut-PSCs were recorded with Axoscope software version 8.1 (Axon Instruments) and analyzed offline with Mini Analysis Program version 6.0 (Synaptosoft).

**Single GABA<sub>A</sub> channel recordings** were performed at P1, P7 and P15 visually identified hippocampal CA3 pyramidal cells in cell-attached configuration using Axopatch-200A amplifier and pCLAMP acquisition software (Axon Instruments, Union City, CA). Data were low-pass filtered at 2 kHz and acquired at 10 kHz. The glass recording electrodes (4-7 MΩ) were filled with a solution containing (in mM) for recordings of single GABA<sub>A</sub> channels: NaCl 120, KCl 5, TEA-Cl 20, 4-aminopyridine 5, CaCl<sub>2</sub> 0.1, MgCl<sub>2</sub> 10, glucose 10, HEPES-NaOH 10. The pH of pipette solutions was adjusted to 7.2 and the osmolality to 280 mOsmol l<sup>-1</sup>. Analysis of currents trough single channels and current-voltage relationships were performed using Clampfit 9.2 (Axon Instruments) as described by (58).

**Morphological analysis**

During electrophysiological recordings, biocytin (0.5%, Sigma, USA) was added to the pipette solution for post hoc reconstruction. Images were acquired using a Leica SP5 X confocal microscope, with a 40x objective and 0.5 µm z-step. Neurons were reconstructed tree-dimensionally using Neurolucida software version 10 (MBF Bioscience) from 3D stack images. The digital reconstructions were analyzed with the software L-Measure to measure the number of primary branches and the total number of ramifications of each neuron (59). Comparisons between groups were done directly in L-Measure.

**Immunohistochemistry and quantification**

WT and mutant mice were deeply anaesthetized with intraperitoneal injection of the ketamine/xylazine mixture and transcardially perfused with 0.9% NaCl saline followed by Antigenfix (Diapath, cat #P0014). Brains were post-fixed in Antigenfix overnight at 4°C and included in agar 4%. 50 µm-thick coronal sections were sliced using a vibratom (Zeiss) and stored in PBS at 4°C. Floating slices (of the hippocampal region corresponding to slices 68 to 78 on Allan Brain Atlas) were incubated for 1 hour with blocking solution containing 0.1% (v/v) Triton X-100, 10% (v/v) normal goat serum (NGS) in PBS, at room temperature. Sections were then incubated with primary antibodies diluted in incubation solution (0.1% (v/v) Triton X-100,
3% (v/v) NGS, in PBS), overnight at 4C°. After 3 x 10 min washes in PBS, brain sections were incubated with secondary antibodies diluted in the incubation solution, for 2 hours at RT. Sections were washed 3 x 10 min in PBS and mounted in Fluoromount-G (EMS, cat #17984-25). Primary antibodies used were: rabbit polyclonal anti-cFos (1:5000, Santa Cruz Biotech , cat #ab190289), goat polyclonal anti-Sst (D20) (1:500, Santa Cruz Biotech, cat #sc-7819), mouse monoclonal anti-Sst (H-11) (1:500, Santa Cruz Biotech, cat #sc-74556), goat polyclonal anti-PV (1:6000, SWANT, cat #PVG213). Fluorochrome-conjugated secondary antibodies used were: goat anti-rabbit Alexa Fluor 647 (1:500, Invitrogen, cat # A32733), goat anti-rabbit Alexa Fluor 488 (1:500, Invitrogen, cat #A-31565), goat anti-mouse Alexa Fluor 488 (1:500, Invitrogen, cat #A21121), donkey anti-goat Alexa Fluor 488 (1:500, Invitrogen, cat # A32814).

For c-Fos, PV and SST quantification, images were acquired using a fluorescence microscope (Zeiss Axioplan 2 microscope with an Apotome module), and z stacks of 8 µm were performed for each section. Counting were performed on the right and left hippocampus for 5-7 sections (cFos) or 7-9 sections (PV, SST) per animal in the hippocampal regions indicated on the figures and corresponding to slices 68 to 78 on Allen Brain Atlas.

**OT binding assay**

Adult WT and mutant mice were sacrificed and non-perfused mouse brain were frozen in -25°C isopenthanne and stored at -80°C until cut. 14 µm thick brain slices were cut using a cryostat (Frigocut-2700, Reichert-Jung) and collected on chromallume-coated slides and stored at -80°C until use. Slides were pre-incubated for 5 minutes in a solution of 0.2% paraformaldehyde in phosphate-buffered saline (pH 7.4), and rinsed twice in 50 mM Tris HCl + 0.1% BSA buffer. Slides were then put in a humid chamber and covered with 400 µL of incubation medium (50 mM Tris HCl, 0.025% bacitracin, 5 mM MgCl2, 0,1% BSA) containing the radiolabeled I [125] OVTA (Perkin Elmer), at a concentration of 10 pM. After a 2h incubation under gentle agitation, the incubation medium is removed and slides are rinsed twice in ice-cold incubation medium and a third time in ice cold distilled water. Each slide is then dried in a stream of cold air, and placed in a X-ray cassette in contact with a KODAK film for 3 days.

ROIs were chosen and analyzed through ImageJ, using Paxinos’ Mouse Brain Atlas as a reference to find the brain areas of interest. To remove background noise caused by nonspecific binding, each slide was compared with its contiguous one, which had been incubated in presence of an excess of “cold” oxytocin (2 µM). Net grey intensity was quantified and then converted to nCi/mg tissue equivalent using a calibration curve. For each region, a minimum of 4 slices per brain were included in the analysis. Data plotted on graphs are the
differences between the total and the nonspecific binding. Right and left hemispheres were kept separate.

**Chromogenic In situ Hybridization**

Fresh-frozen brains from WT mice at post-natal days (P) 7, P21, and P28 were sectioned in a cryostat in the coronal plane at 20μm thickness and mounted on Superfrost Plus slides and stored at -80°C. RNA detection was performed on tissue sections using RNAscope 2.5HD Duplex Assay (Cat #322430, Advanced Cell Diagnostics (ACD), Hayward, CA). The two probes used are synthetic oligonucleotide probes complementary to the nucleotide sequence 1198 – 2221 of Oxtr (NM_001081147.1) (Oxtr-E4-C2, ACD Cat #411101-C2) and 3229 – 4220 of Magel2 (NM_013779.2) (Magel2-01, ACD Cat #535901). Briefly, slides were fixed in 4% paraformaldehyde in PBS (pH 9.5) on ice for 2 hours and dehydrated in increasing concentrations of alcohol, then stored in 100% ethanol overnight at -20°C. The slides were air dried for 10 minutes, then pretreated in target retrieval solution (ref. 322001, ACD) for 5 minutes while boiling, after which, slides were rinsed 2 times in water followed by 100% ethanol and then air dried. A hydrophobic barrier pen (ImmEdge) was used to create a barrier around selected sections. Selected sections were then incubated with protease plus (ref. 322331, ACD) for 15 minutes in a HybEZ oven (ACD) at 40°C, followed by water washes. The sections were then hybridized with the probe mixture at 40°C for 2 hr per slide. Unbound hybridization probes were removed by washing 2 times in wash buffer. After hybridization, sections were subjected to signal amplification using the HD 2.5 detection Kit following the kit protocol. Hybridization signal was detected using a mixture of fast-RED solutions A and B (60:1) for Oxtr-E4-C2 and a mixture of Fast-GREEN solutions A and B (50:1) for Magel2-01. The slides were then counterstained with Gill’s hematoxylin and air-dried in a 60°C oven for 15 min. Slides were cooled and cover-slipped with Vectamount TM (Vector Laboratories, Inc. Burlingame, CA). Slides were imaged at 4x and 20x on a bright field microscope (Keyence BZ-X710, Keyence Corp., Osaka, Japan). Hippocampal sections were investigated for colocalization of Oxtr (red) with Magel2 (blue-green) transcripts.

**Western Blot**

P7 mice were sacrificed and hippocampi were dissected and rapidly frozen in liquid nitrogen and stored at -80°C until protein extraction. Hippocampi were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) Triton-100, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, and protease inhibitors (complete protease inhibitor cocktail tablets, Roche, 1 tablet per 50 mL) and protein concentrations were determined following centrifugation of the lysate at 16,000 x g at 4 °C for 20 minutes using the Bradford method with
bovine serum albumin as the standard. Tissue lysates (15 µg) in SDS sample buffer (1X NuPAGE LDS sample buffer (Invitrogen), containing 1% (v/v) 2-mercaptoethanol) were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 30 min with TBS-Tween buffer (TTBS, Tris/HCl, pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween-20) containing 5% (w/v) skim milk. The membranes were then immunoblotted in 5% (w/v) skim milk in TTBS with the indicated primary antibodies overnight at 4°C. The blots were then washed six times with TTBS and incubated for 1 hour at room temperature with secondary HRP-conjugated antibodies diluted 5000-fold in 5% (w/v) skim milk in TTBS. After repeating the washing steps, the signal was detected with the enhanced chemiluminescence reagent. Immunoblots were developed using ChemiDoc™ Imaging Systems (Bio-Rad). Primary antibodies used were: anti-KCC2 phospho-Ser940 (Thermo Fisher Scientific, cat #PA5-95678), anti-KCC2 phospho-Thr1007 (Thermo Fisher Scientific, cat #PA5-95677), anti-Pan-KCC2, residues 932-1043 of human KCC2 (NeuroMab, cat #73-013), anti(neuronal)-β-Tubulin III (Sigma-Aldrich, cat #T8578). Horseradish peroxidase-coupled (HRP) secondary antibodies used for immunoblotting were from Pierce. Figures were generated using Photoshop and Illustrator (Adobe). The relative intensities of immunoblot bands were determined by densitometry with ImageJ software.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Prism 7.0 software, Inc, La Jolla, CA, USA). All statistical tests were two-tailed and the level of significance was set at P<0.05. Appropriate tests were conducted depending on the experiment; tests are indicated in the figure legends or detailed in supplementary statistical file. Values are indicated as Q2 (Q1, Q3), where Q2 is the median, Q1 is the first quartile and Q3 is the third quartile when non-parametric tests were performed and scatter dot plots report Q2 (Q1,Q3) or as mean ± SEM when parametric tests were performed usually in histograms. N refers to the number of animals or primary culture preparations, while n refers to the number of brain sections or hippocampi or cells recorded.

Mann-Whitney (MW) non-parametric test or t-test (parametric test) were performed to compare two matched or unmatched groups. ANOVA or Kruskal-Wallis tests were performed when the different groups have been experienced in the same experimental design only; if this was not the case, MW or t-test were used. One-way ANOVA followed by Bonferroni or Dunnett’s or Tukey’s post-hoc tests were used to compare three or more independent groups. Two-way ANOVA followed by Bonferroni post-hoc test was performed to compare the effect of two factors on unmatched groups. *: p< 0.05; **: p <0.01; ***: p<0.001; ****: p<0.0001. All the statistical analyses (corresponding to each figure) are reported in a specific file.
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Supplementary data: 7 supplementary figures and Statistical file including statistical analysis for each figure.
REFERENCES

1. Boccaccio I, Glatt-Deeley H, Watrin F, Roeckel N, Lalande M, Muscatelli F. The human MAGEL2 gene and its mouse homologue are paternally expressed and mapped to the Prader-Willi region. Hum Mol Genet. 1999;8(13):2497-505.
2. Schaaf CP, Gonzalez-Garay ML, Xia F, Potocki L, Gripp KW, Zhang B, et al. Truncating mutations of MAGEL2 cause Prader-Willi phenotypes and autism. Nat Genet. 2013.
3. Fountain MD, Schaaf CP. Prader-Willi Syndrome and Schaaf-Yang Syndrome: Neurodevelopmental Diseases Intersecting at the MAGEL2 Gene. Diseases. 2016;4(1).
4. Schaller F, Watrin F, Sturny R, Massacrier A, Szepetowski P, Muscatelli F. A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene. Hum Mol Genet. 2010;19(24):4895-905.
5. Meziane H, Schaller F, Bauer S, Villard C, Matarazzo V, Riet F, et al. An Early Postnatal Oxytocin Treatment Prevents Social and Learning Deficits in Adult Mice Deficient for Magel2, a Gene Involved in Prader-Willi Syndrome and Autism. Biol Psychiatry. 2015;78(2):85-94.
6. Fountain MD, Aten E, Cho MT, Juusola J, Walkiewicz MA, Ray JW, et al. The phenotypic spectrum of Schaaf-Yang syndrome: 18 new affected individuals from 14 families. Genet Med. 2017;19(1):45-52.
7. Dai YC, Zhang HF, Schon M, Bockers TM, Han SP, Han JS, et al. Neonatal Oxytocin Treatment Ameliorates Autistic-Like Behaviors and Oxytocin Deficiency in Valproic Acid-Induced Rat Model of Autism. Frontiers in cellular neuroscience. 2018;12:355.
8. Penagarikano O, Lazaro MT, Lu XH, Gordon A, Dong H, Lam HA, et al. Exogenous and evoked oxytocin restores social behavior in the Cntnap2 mouse model of autism. Science translational medicine. 2015;7(271):271ra8.
9. Francis SM, Sagar A, Levin-Decanini T, Liu W, Carter CS, Jacob S. Oxytocin and vasopressin systems in genetic syndromes and neurodevelopmental disorders. Brain Res. 2014.
10. Mansouri M, Pouretemad H, Roghani M, Wegener G, Ardalan M. Autistic-Like Behaviours and Associated Brain Structural Plasticity are Modulated by Oxytocin in Maternally Separated Rats. Behav Brain Res. 2020:112756.
11. Johnson ZV, Walum H, Xiao Y, Riefkohl PC, Young LJ. Oxytocin receptors modulate a social salience neural network in male prairie voles. Horm Behav. 2017;87:16-24.
12. Raam T, McAvooy KM, Besnard A, Veenema AH, Sahay A. Hippocampal oxytocin receptors are necessary for discrimination of social stimuli. Nature communications. 2017;8(1):2001.
13. Lin YT, Hsieh TY, Tsai TC, Chen CC, Huang CC, Hsu KS. Conditional Deletion of Hippocampal CA2/CA3a Oxytocin Receptors Impairs the Persistence of Long-Term Social Recognition Memory in Mice. J Neurosci. 2018;38(5):1218-31.
14. Okuyama T. Social memory engram in the hippocampus. Neurosci Res. 2018;129:17-23.
15. Cilz NI, Cymerblit-Sabbage A, Young WS. Oxytocin and Vasopressin in the Rodent Hippocampus. Genes Brain Behav. 2018:e12535.
16. Young WS, Song J. Characterization of Oxytocin Receptor Expression Within Various Neuronal Populations of the Mouse Dorsal Hippocampus. Frontiers in molecular neuroscience. 2020;13:40.
17. Hammock EA. Developmental perspectives on oxytocin and vasopressin. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2015;40(1):24-42.
18. Mitre M, Marlin BJ, Schiavo JK, Morina E, Norden SE, Hackett TA, et al. A Distributed Network for Social Cognition Enriched for Oxytocin Receptors. J Neurosci. 2016;36(8):2517-35.
19. Tirko NN, Eyring KW, Carcea I, Mitre M, Chao MV, Froemke RC, et al. Oxytocin Transforms Firing Mode of CA2 Hippocampal Neurons. Neuron. 2018;100(3):593-608 e3.
20. Ripamonti S, Ambrozkiewicz MC, Guzzi F, Gravati M, Biella G, Bormuth I, et al. Transient oxytocin signaling primes the development and function of excitatory hippocampal neurons. eLife. 2017;6.
21. Tauber M, Boulanour K, Diene G, Cabal-Berthoumieu S, Ehlinger V, Fichaux-Bourin P, et al. The Use of Oxytocin to Improve Feeding and Social Skills in Infants With Prader-Willi Syndrome. Pediatrics. 2017.
22. Matarazzo V, Muscatelli F. Natural breaking of the maternal silence at the mouse and human imprinted Prader-Willi locus: A whisper with functional consequences. Rare diseases. 2013;1:e27228.
23. Zhang JB, Chen L, Lv ZM, Niu XY, Shao CC, Zhang C, et al. Oxytocin is implicated in social memory deficits induced by early sensory deprivation in mice. Molecular brain. 2016;9(1):98.
24. Karlsson SA, Haziri K, Hansson E, Kettunen P, Westberg L. Effects of sex and gonadectomy on social investigation and social recognition in mice. BMC neuroscience. 2015;16:83.
25. Leonzino M, Busnelli M, Antonacci F, Verderio C, Mazzanti M, Chini B. The Timing of the Excitatory-to-Inhibitory GABA Switch Is Regulated by the Oxytocin Receptor via KCC2. Cell reports. 2016;15(1):96-103.
26. Ben-Ari Y. Is birth a critical period in the pathogenesis of autism spectrum disorders? Nat Rev Neurosci. 2015;16(8):498-505.
27. Tzio R, Minlebaev M, Rheims S, Ivanov A, Jorquera I, Holmes GL, et al. Postnatal changes in somatic gamma-aminobutyric acid signalling in the rat hippocampus. Eur J Neurosci. 2008;27(10):2515-28.
28. Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamka K, et al. The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature. 1999;397(6716):251-5.
29. Zhang J, Cordshagen A, Medina I, Nothwang HG, Wisniewski JR, Winkelhofer M, et al. Staurosporine and NEM mainly impair WNK-SPAK/OSR1 mediated phosphorylation of KCC2 and NKCC1. PLoS One. 2020;15(5):e0232967.
30. Kahle KT, Merner ND, Friedel P, Silayeva L, Liang B, Khanna A, et al. Genetically encoded impairment of neuronal KCC2 cotransporter function in human idiopathic generalized epilepsy. EMBO Rep. 2014;15(7):766-74.
31. Friedel P, Kahle KT, Zhang J, Hertz N, Pisella LJ, Buhler E, et al. WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. Sci Signal. 2015;8(383):ra65.
32. de Los Heros P, Alessi DR, Gourlay R, Campbell DG, Deak M, Macartney TJ, et al. The WNK-regulated SPAK/OSR1 kinases directly phosphorylate and inhibit the K+/Cl- co-transporters. Biochem J. 2014;458(3):559-73.
33. Lee HH, Deeb TZ, Walker JA, Davies PA, Moss SJ. NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents. Nat Neurosci. 2011;14(6):736-43.
34. Caldwell HK, Aulino EA, Freeman AR, Miller TV, Witchey SK. Oxytocin and behavior: Lessons from knockout mice. Developmental neurobiology. 2017;77(2):190-201.
35. Kenkel WM, Perkeybile AM, Yee JR, Pournajafi-Nazarloo H, Lillard TS, Ferguson EF, et al. Behavioral and epigenetic consequences of oxytocin treatment at birth. Sci Adv. 2019;5(5):eaav2244.
36. Ates T, Oncul M, Dilsiz P, Topcu IC, Civas CC, Alp MI, et al. Inactivation of Magel2 suppresses oxytocin neurons through synaptic excitation-inhibition imbalance. Neurobiol Dis. 2019;121:58-64.
37. Tacer KF, Potts PR. Cellular and disease functions of the Prader-Willi Syndrome gene MAGEL2. Biochem J. 2017;474(13):2177-90.
38. Kahle KT, Deeb TZ, Puskarjov M, Silayeva L, Liang B, Kaila K, et al. Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. Trends Neurosci. 2013;36(12):726-37.
39. Pisella LI, Gaiarsa JL, Diabira D, Zhang J, Khalilov I, Duan J, et al. Impaired regulation of KCC2 phosphorylation leads to neuronal network dysfunction and neurodevelopmental pathology. Sci Signal. 2019;12(603).
40. Ben-Ari Y, Spitzer NC. Nature and nurture in brain development. Trends Neurosci. 2004;27(7):361.
41. Leinekugel X, Khazipov R, Cannon R, Hirase H, Ben-Ari Y, Buzsaki G. Correlated bursts of activity in the neonatal hippocampus in vivo. Science. 2002;296(5575):2049-52.
42. Shi Y, Grieco SF, Holmes TC, Xu X. Development of Local Circuit Connections to Hilar Mossy Cells in the Mouse Dentate Gyrus. eNeuro. 2019;6(2).
43. He Q, Nomura T, Xu J, Contractor A. The Developmental Switch in GABA Polarity Is Delayed in Fragile X Mice. Journal of Neuroscience. 2014;34(2):446-50.
44. Ben-Ari Y. The GABA excitatory/inhibitory developmental sequence: a personal journey. Neuroscience. 2014;279:187-219.
45. Banerjee A, Rikhye RV, Breton-Provencher V, Tang X, Li C, Li K, et al. Jointly reduced inhibition and excitation underlies circuit-wide changes in cortical processing in Rett syndrome. Proc Natl Acad Sci U S A. 2016.
46. Kang E, Song J, Lin Y, Park J, Lee JH, Hussani Q, et al. Interplay between a Mental Disorder Risk Gene and Developmental Polarity Switch of GABA Action Leads to Excitation-Inhibition Imbalance. Cell reports. 2019;28(6):1419-28 e3.
47. Sohal VS, Rubenstein JLR. Excitation-inhibition balance as a framework for investigating mechanisms in neuropsychiatric disorders. Mol Psychiatry. 2019;24(9):1248-57.
48. Harrington AJ, Riaiisi A, Rajkovich K, Berto S, Kumar J, Molinaro G, et al. MEF2C regulates cortical inhibitory and excitatory synapses and behaviors relevant to neurodevelopmental disorders. eLife. 2016;5.
49. Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM, et al. A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. Science. 2007;318(5847):71-6.
50. Unichenko P, Yang JW, Kirischuk S, Kolbaev S, Kilb W, Hammer M, et al. Autism Related Neuroligin-4 Knockout Impairs Intracortical Processing but not Sensory Inputs in Mouse Barrel Cortex. Cerebral cortex. 2017;1-14.
51. Wood L, Shepherd GM. Synaptic circuit abnormalities of motor-frontal layer 2/3 pyramidal neurons in a mutant mouse model of Rett syndrome. Neurobiol Dis. 2010;38(2):281-7.
52. Carter CS. Developmental consequences of oxytocin. Physiol Behav. 2003;79(3):383-97.
53. Maldonado P, Nuno-Perez, A., Kirchner, J., Hammock, E., Gjorgjieva, J. and Lohmann, C. Oxytocin shapes spontaneous activity patterns in the developing visual cortex by activating somatostatin interneurons. bioRxiv. 2020.
54. Perez SM, Boley A, Lodge DJ. Region specific knockdown of Parvalbumin or Somatostatin produces neuronal and behavioral deficits consistent with those observed in schizophrenia. Translational psychiatry. 2019;9(1):264.
55. Scoggin D, Manago F, Maltese F, Bruni S, Nigro M, Dautan D, et al. Somatostatin interneurons in the prefrontal cortex control affective state discrimination in mice. Nat Neurosci. 2020;23(1):47-60.
56. Smith C, Kingsbury, M., Dziabis, J., Hanamsagar, R., Malacon, K. Tran,J., Norris, A., Gulino, M. and Bilbo, S. Neonatal immune challenge induces female-specific changes in social behavior and somatostatin cell number, independent of microglial inflammatory signaling bioRxiv. 2020.
57. Kaech S, Banker G. Culturing hippocampal neurons. Nature protocols. 2006;1(5):2406-15.
58. Tizio R, Ivanov A, Bernard C, Holmes GL, Ben-Ari Y, Khazipov R. Membrane potential of CA3 hippocampal pyramidal cells during postnatal development. J Neurophysiol. 2003;90(5):2964-72.
59. Scorcioni R, Polavaram S, Ascoli GA. L-Measure: a web-accessible tool for the analysis, comparison and search of digital reconstructions of neuronal morphologies. Nature protocols. 2008;3(5):866-76.
(A) Paradigm of the three-chamber test. Sniffing time between mice is measured in each test. (B) WT males (N=9) show normal behavior in all the steps of the test; Magel2 KO males (N=9) show a significant impairment in short term social memory. (C) WT mice were treated in the first week of life with vehicle or OT and then tested at four months. WT mice treated with vehicle (N=18) or treated with OT (N=10) have similar profiles with significant differences in each step of the test. (D) Magel2 KO mice were treated in the first week of life with vehicle or OT and then tested at four months. Magel2 KO-vehicle (N=19) mice show a significant difference in the social exploration and social discrimination, but, in short term social memory, they do not show a higher sniffing time with the novel mouse. OT-treated Magel2 KO (N=19) mice present significant differences in each step of the step. Data represented in histograms report the interaction time (time of sniffing in seconds) as mean ± SEM. Mann-Whitney Test. *P<0.05, **P<0.01, ***P<0.001. Statistical analysis is reported in Supplemental Table 1.
Figure 2. cFos activity in aCA2/CA3d and aDG regions of Magel2 KO and WT male mice following the social memory task in the three-chamber test.

(A) Paradigm of the three-chamber test (+SI) followed 90 min later by dissection of the brains and immunohistochemistry experiments. Control mice (-SI) were not tested in the three-chamber test. (B-C) cFos-immunolabeling on coronal brain sections in the aCA2/CA3d and aDG regions as indicated in (B) of WT-SI, WT+SI and Magel2 KO+SI mice (C). (D-E) Quantification of cFos+ cells/section in WT-SI (n=46, N=6), WT+SI (n=24, N=4), Magel2 KO-SI (n=38, N=4) and Magel2 KO+SI (n=24, N=4) in the aCA2/CA3d region (D) and in the aDG region (E).

N: number of animals, n: number of sections/hippocampus. Scale bar: 500μm (B); 100μm (C).

Data represented in box and whisker-plots report the number of cFos + cells by sections (6 sections/hippocampus) with the median (Q2) and quartiles (Q1, Q3) for the genotype and treatment. One-way ANOVA + Dunnett’s post hoc test, ***P<0.001.

Statistical analysis is reported in Supplemental Table 2.
Figure 3: Expression of Magel2 and Oxytocin receptor (Oxtr) transcripts in hippocampus of wild-type male mice at P7 and P21.

(A) Representative image obtained by RNAscope technology showing the respective localization of Magel2 (blue) and Oxtr (pink) transcripts in dentate gyrus (DG) and aCA2/CA3d region of hippocampus. (B) Representative image obtained by RNAscope technique showing the respective localization of Sst (blue) and Oxtr (pink) transcripts in the aCA2/CA3 region from hippocampal slices of WT male pups at P10. Arrows indicate colocalization of both transcripts in the same cell. Scale bar: 100 µm.
Figure 4: Quantification of OT binding sites by brain autoradiography in Magel2 KO male mice treated with OT or vehicle versus WT-vehicle male mice.

(A-C-E) Representative sections of autoradiographic labeling of OT binding sites displayed in grayscale, showing the regions of interest (ROI) selected for analysis: (A) anterior CA2/CA3 (aCA2/CA3), (C) dentate gyrus (aDG) and (E) ventral CA1/CA2/CA3 (vCA1/CA2/CA3) regions of hippocampus. (B-D-F) Quantification of OT binding sites expressed as nCi/mg of tissue equivalent in (B) anterior CA2/CA3, (D) dentate gyrus and (F) ventral CA1/CA2/CA3 regions of hippocampus. Histograms report median (Q2) and quartiles (Q1, Q3). OT binding sites in nCi/mg of tissue equivalent. 3 (N) mice and 6 (n) hippocampi have been analyzed for each group. Data represented in box and whisker-plots report the quantity of radiolabeling by hippocampus, with scattered plots that show individual data points. One-way ANOVA + Bonferroni post hoc test, **P<0.01, ****P<0.0001. Scale Bar: 3 mm. Statistical analysis is reported in Table 4.
Figure 5: Quantification of somatostatin (SST) immunopositive cells in the anterior hippocampus region of Magel2 KO adult mice having or not been treated by OT in the first week of life and compared with WT mice.

(A-L) Immunolabeling on coronal hippocampal sections at adulthood in WT (A,C,E) and Magel2 KO (B,D,F), and in WT (G,I,K) and Magel2 KO+OT (H,J,L) with a magnification in the aCA2/CA3d region (C,D,I,J) and in the DG region (E,F,K,L) in which the SST+ cells are counted. (M-P) Number of SST+ cells by section in both aCA2/CA3d (M,O) and aDG (N,P) and comparing WT (N=4, n=48) with Magel2 KO (N=4, n=48) animals (M,N) or WT (N=3, n=36) with Magel2 OT+ (N=5, n=56) (O,P) mice. N: number of animals, n: number of sections/hippocampus. Data represented in whisker-plots report the number of SST+ cells by section with Q2(Q1, Q3), with scattered plots showing individual data points. Mann-Whitney Test **P<0.01, ***P<0.001. Scale bar (A-H): 500 µm; (C-L): 100 µm.

Statistical analysis is reported in Table 5.
Figure 6: Spontaneous Glutamatergic and GABAergic synaptic activity of CA3 pyramidal neurons in the anterior hippocampus region of Magel2 KO mice versus WT juvenile mice and having been OT-treated or vehicle-treated in neonates.

(A) Paradigm of the test. WT or Magel2 KO mice have been or not injected with OT in the first week of life then neurons are recorded in brain slices at P25. (B) Examples of whole cell recordings performed at a holding potential of -45 mV for each genotype or treatment. The glutamatergic synaptic currents are inwards and the GABAergic synaptic currents are outwards (C-D) Values in the different genotypes and treatment of the Glut-sPSCs frequency (C) and amplitude (D). (E-F) Values in the different genotypes and treatment of the GABA-sPSCs frequency (E) and amplitude (F). Magel2 KO (N= 7, n=16), WT (N=7, n=15), Magel2 KO+OT, (N=5, n=21) and WT+OT (N=4, n=15) have been analyzed, with N: number of mice and n: number of recorded cells. Data represented in whisker-plots report the different values of recorded cells with mean ±SEM, with scattered plots showing individual data points. One-way ANOVA + Tuckey post hoc test. *P<0.05, **P<0.01. Statistical analysis is reported in Supplemental Table 6.
Figure 7. The excitatory-to-inhibitory developmental GABA-shift in Magel2 KO versus WT hippocampi and the effect on an OT-treatment.

(A-B) GABA-induced Ca2+ responses in Magel2 KO developing hippocampal neuronal cultures versus WT.

(A) Percentage of WT and Magel2 KO E18 hippocampal neurons showing GABA-induced Ca2+ responses at selected in vitro days (DIV). (B) Representative traces of [Ca2+]i variations (delta F340/380) in DIV4 WT and Magel2 KO neurons upon 100 μM GABA administration. Data are presented in histograms with mean ± SEM; unpaired t test with Welch’s correction; ****P<0.0001.

(C-D) Measures of the driving force for GABA (DFGABA), using cell-attached recordings of single GABAA channels, of aCA3d pyramidal neurons. (C) Average values of the DFGABA at P1, P7 and P15 in Magel2 KO (P1 N= 3, n= 20; P7: N= 7, n= 56; P15: N= 4, n= 29) versus WT (P1 N= 3, n= 19; P7: N= 6, n= 42; P15: N= 3, n= 23) mice. Data are presented in histograms with mean ± SEM; unpaired t test with Welch’s correction: *P<0.05. (D) Graph reporting the relative changes of the DFGABA at P7 in untreated Magel2-KO mice (N= 7, n= 56) compared with WT mice (N= 6, n= 42) and in OT-treated WT (N= 3, n= 37) and Magel2-KO (N= 4, n= 56) mice compared with WT-vehicle (N= 3, n= 37) mice. N: number of mice and n: number of recorded cells. One-way ANOVA + Dunnett’s post hoc test: **P<0.01.

Statistical analysis is reported in Supplemental Table 7.
Figure 8. Abundance and phosphorylation state of KCC2 in WT and Magel2 KO pups (P7).

(A) Immunoblot analysis of WT (N=5) and Magel2 KO (N=6) hippocampi of P7 mice with pan-KCC2 antibody or phosphorylation site-specific antibodies recognizing P-Ser940 or P-Thr1007 of KCC2. An antibody recognizing neuron-specific β3 tubulin was used to normalize the quantity of proteins. Numbers on the left indicate molecular weight.

(B) Boxplots report band intensities from (A) as Q2(Q1,Q3), with scattered plot showing individual data points. Mann-Whitney test, *P<0.05.

(C) A model of KCC2-dependent control of neuronal Cl− in Magel2 KO pups. At this stage of neuronal development, the surface expression of KCC2, that determines its ion-transport activity, depends on the ratio of reciprocal phosphorylation of its Ser940 and Thr1007 residues. The Ser940 phosphorylation increases KCC2’s cell surface stability, whereas the Thr1007 phosphorylation exerts opposite to Ser940 effect and favors internalization (shown with brown arrows). Compared to WT, the CA3 neurons in hippocampi from Magel2 KO mice are characterized by depolarizing action of GABA (e.g. activation of GABA generates Cl− efflux) that reflects higher [Cl−]. In Magel2 KO hippocampi the amount of KCC2’s Ser940 phosphorylation is significantly lower as compared to WT hippocampi whereas the amount of phosphorylated Thr1007 remains unchanged. Respectively, the decreased P-Ser940/P-Thr1007 ratio results in predominance of KCC2 internalization over surface expression. As consequence of the decreased amount of surface expressed molecules, the Cl− extrusion ability of KCC2 is decreased that causes increase of [Cl−]i and depolarizing shift of GABA.

The model includes also important components that are known to control the level of Ser940 and Thr1007 phosphorylation. The Ser940 is directly phosphorylated by kinase C (pkC) and dephosphorylated under pathology conditions by protein phosphatase type 1 (PP1). The Thr1007 is directly phosphorylated by SPAK. It remains to be elucidated whether in Magel2 KO mice the decreased level of Ser940 results from reduction of pkC activity or enhancement of PP1 action. Statistical analysis is reported in Supplemental Table 8.
Figure 1-figure supplement 1. Social-index values comparing Magel2 KO versus WT male mice or Magel2 KO+OT versus Magel2 KO-vehicle male.

These indexes report for the social exploration: the sniffing time with S1/ sniffing time with S1 + time in empty room x 100; for the discrimination: the sniffing time with S2/ sniffing time with S1 + sniffing time with S2 time x 100 and for short term memory: the sniffing time with S3/ sniffing time with S1 + sniffing time with S3 time x 100. They are measured in (A) Magel2 KO versus WT mice and (B) Magel2 KO-vehicle and Magel2 KO+OT. It appears clearly that social exploration and social discrimination are similar between WT and Magel2 KO (A) and between Magel2 KO-vehicle and Magel2 KO+OT (B). Only short memory index is decreased in Magel2 KO compared with WT and increased in Magel2 KO+OT compared with Magel2 KO-vehicle. Data in histograms report social indexes calculated for each individual as mean ± SEM. Mann-Whitney test, *P<0.05.

Statistical analysis is reported in Supplemental Table 1- Supplement 1.
Figure 1-figure supplement 2. Social behavior in three-chamber test of female Magel2 KO adults versus WT adults.
(A) Paradigm of the three-chamber test. Sniffing time between mice is measured in each test. (B) WT (N=11) females do present differences in social exploration but do not present significant differences in social discrimination and short-term social memory, suggesting that the three-chamber test is not relevant to assess the social behavior in females. Similarly, Magel2 KO (N=9) females do not present significant differences in all three steps of the paradigm. Data in histograms report interaction time (time of sniffing in seconds) as mean ± SEM. Mann-Whitney test, *P<0.05. Statistical analysis is reported in Supplemental Table 1- Supplement 2.
Figure 1-figure supplement 3. Behavioral tests in male and female WT mice having been OT-treated or vehicle-treated in neonates.

Behavioral analysis of male and female OT-treated versus vehicle-treated WT mice. (A) Novel object recognition (NOR) test allows to assess the non-social memory. Training simply involves visual exploration of two identical objects, while after one hour the test session involves replacing one of the previously explored objects with a novel object. Because mice have an innate preference for novelty, a mouse that remembers the familiar object (same object) will spend more time exploring the novel object (new object) conducting to a discrimination index significantly different from 50%. A similar discrimination index is observed in vehicle or OT-treated WT males and females. (B) Open field (OF) test measures locomotor activity and vertical activity (rearing) and anxiety-related behavior (time in zones and grooming): no significant differences have been detected in all those activities between OT-treated and vehicle-treated WT mice but WT+OT females present an approximately 15% reduction in the moved distance and a tendency to increase rearing. (C) Elevated Plus Maze (EPM) test allows to measure anxiety (time spent in open arms and number of entries in open arms) and shows a similar behavior with a tendency to spend more time and enter more often in open arms in WT+OT males, suggesting an anxiolytic effect of OT. Data in histograms report mean ± SEM. Mann-Whitney test, * P<0.05.

Statistical analysis is reported in Supplemental Table 1- Supplement 3.
Figure 5-figure supplement 1: Quantification of parvalbumin (PV) immunopositive cells in the anterior hippocampus region of Magel2 KO adult mice compared with WT mice.

(A-D) PV immunolabeling on coronal hippocampal sections at the level of aCA2/CA3d and aDG regions in WT (A-C) and Magel2 KO (B-D). (E-F) Number of PV+ cells by section in both aCA2/CA3d (E) and aDG (F) and comparing WT (N=4) with Magel2 KO (N=4) mice (M-N). Scale bar: 100µm. Data represented in whisker-plots report the number of PV+ cells by sections (8 sections/ hippocampus) with Q2(Q1, Q3) for each genotype and scattered plots showing individual data points. Mann-Whitney Test.
Figure 6-figure supplement 1. Miniatures Glutamatergic and GABAergic synaptic activity of aCA3 pyramidal neurons in the anterior hippocampus region of Magel2 KO juvenile mice compared with WT.

(A) Representative whole-cell recordings of miniature Glutamatergic (inward) and GABAergic (outward) currents (holding potential=–45mV) in juvenile WT and Magel2 KO aCA3 pyramidal neurons. (B-E) The amplitudes (B and D) and frequencies (C and E) of the miniatures glutamatergic (B and C) and GABAergic (D and E) postsynaptic currents. GABAergic frequency (Hz) (WT n=16; Magel2 KO n=17), GABAergic amplitude (pA) (WT n=16; Magel2 KO n=17) and glutamatergic frequency (WT n=14; Magel2 KO n=18) are similar but glutamatergic amplitude (pA) is significantly different. WT (N=5, n=14); Magel2 KO (N=5, n=18). N: number of mice and n: number of recorded cells. Data represented in histograms are mean ± SEM. Mann Whitney test, ***P<0.001.

Statistical analysis is reported in Supplemental Table 6-Supplement 1.
Figure 6—figure supplement 2. Morphology of aCA3 recorded pyramidal neurons in Magel2 KO versus WT mice. (A) Representative distribution of apical and (B) basal dendritic complexity obtained with Sholl analysis. (C) Quantification of total length of the apical and (D) basal dendrites. (E) Quantification of the mean number of bifurcations in apical and (F) basal dendrites. (G) Quantification of the mean number of total branches in apical and (H) basal dendrites following 3D reconstructions from CA3 pyramidal neurons recorded in previous experiments (Figure 6). (I) Representative WT and Magel2 KO reconstructed CA3 pyramidal neurons. Histograms indicate Q2(Q1, Q3). Each dot represents a neuron (n). WT (N=3, n=7), Magel2 KO (N=3, n=11). N: number of mice and n: number of recorded cells. Mann-Whitney test. Statistical analysis is reported in Supplemental Table 6—Supplement 2.
Figure 7-Supplement 1. Parameters to validate the in vitro Calcium imaging analysis and the DFGABA study in Magel2 KO versus WT mice.

(A) Amplitude of GABA-induced and (B) KCl-induced Ca\textsuperscript{2+} peaks in WT and Magel2 KO E18 neurons at the different DIVs. Number of responsive cells is reported in the bars. Data are from two to five different preparations, 6-8 embryos each preparation (six to fifteen coverslips analyzed).

(C-F) Electrophysiological parameters of pyramidal cells (PCs) and interneurons (INs) recorded at P7 in Magel2 KO versus WT CA3 region of hippocampus. (C) Driving force for GABA (DFGABA) measured with single GABAA channels in cell-attached mode in INs and PCs. (D) Resting membrane potential (RMP) of INs and PCs measured in whole-cell current-clamp mode. (E) Cell capacitance of recorded neurons. (F) Conductance of the GABAA channels recorded in cell-attached mode (presented in Figure 8C). Histograms report mean ± SEM. Unpaired t test with Welch’s correction: *P<0.05, **P<0.01, ****P<0.0001.

Statistical analysis is reported in Supplemental Table 7-Supplement 1.
| Figure | Parameter | Test specifications | Genotype | Treatment | Sex | Number of individuals | Mean ± SEM | Statistical test | Median (Q1,Q3) | p-value |
|--------|-----------|---------------------|----------|-----------|-----|-----------------------|------------|------------------|---------------|---------|
| 1B     | Interaction time (sniffing in seconds) | Social exploration | S1       | Empty     | male | 12                     | 78.0 ± 7.7 | paired t-test    | 73.48 (52.34, 100.58) | p=0.00134 |
|        |           |                     | S1       | WT        | male | 34.5 ± 6.6            | 33.7 ± 4.7 | paired t-test    | 27.52 (16.72, 56.22)  | p<0.001  |
|        |           |                     | S1       | Magel2 KO | male | 70.7 ± 7.4            | 28.0 ± 6.0 | paired t-test    | 28.04 (21.00, 45.96)  | p<0.001  |
|        |           | Social discrimination | S1       | Empty     | male | 77.2 ± 16.3          | 68.1 ± 6.8 | paired t-test    | 70.68 (53.50, 89.12)  | p=0.00129 |
|        |           |                     | S1       | WT        | male | 31.5 ± 5.0            | 30.0 ± 4.3 | paired t-test    | 24.64 [21.32, 44.88]  | p=0.004   |
|        |           |                     | S1       | Magel2 KO | male | 43.0 ± 7.1            | 38.2 ± 6.4 | paired t-test    | 41.88 (30.00, 65.74)  | p<0.001  |
|        |           | Social discrimination | S2       | WT        | male | 61.0 ± 7.6            | 63.8 ± 7.4 | Wilcoxon Signed Rank Test | 65.04 (51.80, 81.44)  | p=0.000133 |
|        |           |                     | S2       | Magel2 KO | male | 68.8 ± 9.2            | 67.1 ± 6.4 | Wilcoxon Signed Rank Test | 67.44 [41.44, 97.10]  | p=0.000571 |
|        |           | Social discrimination | S3       | Empty     | male | 68.8 ± 9.2            | 42.0 ± 2.3 | Wilcoxon Signed Rank Test | 44.76 [33.98, 48.36]  | p=0.00001 |
|        |           |                     | S3       | WT        | male | 36.4 ± 4.9            | 84.2 ± 10.5 | Wilcoxon Signed Rank Test | 49.64 [36.72, 63.86]  | p=0.00776 |
|        |           | Social discrimination | S3       | OT        | male | 36.4 ± 4.9            | 84.2 ± 10.5 | Wilcoxon Signed Rank Test | 71.84 [57.14, 103.48] | p=0.00917 |
| 1C     | Interaction time (sniffing in seconds) | Social exploration | S1       | Empty     | male | 18                     | 83.5 ± 6.5 | paired t-test    | 79.48 (70.24, 95.40)  | p=0.00179 |
|        |           |                     | S1       | WT        | male | 49.2 ± 5.6            | 44.2 ± 3.8 | paired t-test    | 45.96 (28.34, 53.86)  | p=0.000291 |
|        |           | Social discrimination | S1       | Magel2 KO | male | 84.5 ± 6.9            | 34.0 ± 3.3 | paired t-test    | 79.16 (58.58, 107.38) | p=0.000571 |
|        |           |                     | S1       | Empty     | male | 67.1 ± 6.4            | 69.3 ± 6.3 | paired t-test    | 67.44 [41.44, 97.10]  | p=0.0001 |
|        |           | Social discrimination | S2       | WT        | male | 42.0 ± 2.3            | 49.9 ± 4.9 | Wilcoxon Signed Rank Test | 44.76 [33.98, 48.36]  | p=0.00001 |
|        |           |                     | S2       | Magel2 KO | male | 84.2 ± 10.5           | 84.2 ± 10.5 | Wilcoxon Signed Rank Test | 49.64 [36.72, 63.86]  | p=0.00776 |
|        |           | Social discrimination | S3       | Empty     | male | 36.4 ± 4.9            | 36.4 ± 4.9 | Wilcoxon Signed Rank Test | 35.08 [27.90, 47.72]  | p=0.00917 |
|        |           |                     | S3       | WT        | male | 36.4 ± 4.9            | 36.4 ± 4.9 | Wilcoxon Signed Rank Test | 71.84 [46.10, 89.52]  | p=0.020   |
| 1D     | Interaction time (sniffing in seconds) | Social exploration | S1       | Empty     | male | 19                     | 65.8 ± 5.1 | paired t-test    | 69.44 (58.72, 79.04)  | p=0.00001 |
|        |           |                     | S1       | Magel2 KO | male | 31.0 ± 3.2            | 29.5 ± 4.3 | paired t-test    | 26.88 (22.80, 40.40)  | p<0.001  |
|        |           | Social discrimination | S1       | Magel2 KO | male | 73.5 ± 7.0            | 38.8 ± 4.0 | paired t-test    | 74.64 (43.12, 92.64)  | p=0.00001 |
|        |           |                     | S1       | Empty     | male | 44.2 ± 3.1            | 49.1 ± 6.0 | paired t-test    | 42.08 (25.36, 52.08)  | p=0.029   |
|        |           | Social discrimination | S2       | Magel2 KO | male | 36.2 ± 4.5            | 34.0 ± 4.7 | paired t-test    | 43.20 [37.52, 50.64]  | p=0.00001 |
|        |           |                     | S2       | Empty     | male | 87.2 ± 6.2            | 32.4 ± 3.8 | paired t-test    | 86.16 [75.12, 98.00]  | p=0.0001 |
|        |           | Social discrimination | S3       | Magel2 KO | male | 56.9 ± 6.5            | 56.9 ± 6.5 | paired t-test    | 53.32 [43.76, 64.80]  | p=0.000702 |

Table 1

Social exploration
Magel2 KO
Social discrimination
Magel2 KO
Short-term social memory
Magel2 KO
Wilcoxon Signed Rank Test
Table 1—Supplement 1, 2, and 3

| Figure | Parameter | Test specifications | Genotype | Treatment | Sex | Number of individuals | Median (Q1,Q3) | Statistical test | p-value |
|--------|-----------|---------------------|-----------|-----------|-----|----------------------|--------------|----------------|---------|
| 1sup1 A | Social index | Social exploration index | S1/Empty | WT | male | 9 | 69.129 (62.504,84.591) | Mann-Whitney | p=0.930 |
|        |           |                     | S2/S1 | Magel2 KO | male | 9 | 20.387 (16.281,24.687) |                     | p=0.596 |
|        |           |                     | S3/S1 | WT | male | 9 | 69.129 (62.504,84.591) |                     | p=0.001 |
| 1sup1 B | Social index | Social exploration index | S1/Empty | Magel2 KO | vehicle | 19 | 31.333 (26.504,51.736) | Mann-Whitney | p=0.048 |
|        |           |                     | S2/S1 | Magel2 KO | OT | 19 | 20.387 (16.281,24.687) |                     | p=0.007 |
|        |           |                     | S3/S1 | Magel2 KO | vehicle | 19 | 31.333 (26.504,51.736) |                     | p=0.021 |
| 1sup2 A | Social index | Social exploration index | S1 | WT | female | 11 | 85.18 ± 11.89 | 89.04 (55.28, 104.48) | paired t-test | p=0.0201 |
|        |           |                     | S2 | WT | female | 12 | 48.85 ± 8.94 | 42.32 (28.40, 75.28) |                     | p=0.0329 |
|        |           |                     | S3 | WT | female | 10 | 43.99 ± 4.82 | 49.76 (31.52, 66.96) |                     | p=0.488 |
|        |           | Social discrimination index | Empty | WT | female | 12 | 48.85 ± 8.94 | 42.32 (28.40, 75.28) |                     | p=0.0329 |
|        |           |                     | Empty | Empty | female | 9 | 59.93 ± 10.28 | 50.24 (30.48, 69.48) |                     | p=0.134 |
|        |           | Social exploration index | S1 | Magel2 KO | female | 9 | 48.85 ± 8.94 | 42.32 (28.40, 75.28) |                     | p=0.0719 |
|        |           |                     | S2 | Magel2 KO | female | 10 | 43.99 ± 4.82 | 49.76 (31.52, 66.96) |                     | p=0.0308 |
|        |           | Short-term social memory index | S1 | Magel2 KO | female | 9 | 48.85 ± 8.94 | 42.32 (28.40, 75.28) |                     | p=0.0334 |
|        |           |                     | S3 | Magel2 KO | female | 10 | 43.99 ± 4.82 | 49.76 (31.52, 66.96) |                     | p=0.0334 |
| 1sup2 B | Social index | Interaction time (sniffing in seconds) | S1 | Vehicle | male | 18 | 26.517 (23.031, 32.118) | Mann-Whitney | p=0.057 |
|        |           |                     | S2 | Vehicle | female | 16 | 31.654 (23.711, 37.174) | Mann-Whitney | p=0.062 |
|        |           |                     | S3 | Vehicle | female | 12 | 22.929 (19.659, 32.465) | Mann-Whitney | p=0.002 |
|        |           | Same object | S1 | Vehicle | male | 16 | 22.929 (19.659, 32.465) | Mann-Whitney | p=0.002 |
|        |           |                     | S2 | Vehicle | female | 12 | 22.929 (19.659, 32.465) | Mann-Whitney | p=0.002 |
|        |           | New object | S1 | Vehicle | male | 16 | 22.929 (19.659, 32.465) | Mann-Whitney | p=0.002 |
|        |           |                     | S2 | Vehicle | female | 12 | 22.929 (19.659, 32.465) | Mann-Whitney | p=0.002 |
| 1sup3 A | NOR | Discrimination index (% of preference) | Same object | Vehicle | male | 10 | 95.822 (92.071, 99.431) | One-Sample Singled Rank Test | p=0.567 |
|        |           |                     | Same object | Vehicle | female | 16 | 46.734 (42.375, 51.092) | One-Sample Singled Rank Test | p=0.062 |
|        |           | New object | Same object | Vehicle | male | 17 | 46.734 (42.375, 51.092) | One-Sample Singled Rank Test | p=0.062 |
|        |           |                     | New object | Vehicle | female | 17 | 46.734 (42.375, 51.092) | One-Sample Singled Rank Test | p=0.062 |
| 1sup3 B | Open Field | Distance moved (in minutes) | Vehicle | OT | male | 18 | 26.517 (23.031, 32.118) | Mann-Whitney | p=0.111 |
|        |           |                     | Vehicle | OT | female | 16 | 31.654 (23.711, 37.174) | Mann-Whitney | p=0.111 |
|        |           | Rearing (number of events) | Vehicle | OT | male | 12 | 22.929 (19.659, 32.465) | Mann-Whitney | p=0.048 |
|        |           |                     | Vehicle | OT | female | 12 | 31.000 (26.000, 36.000) | Mann-Whitney | p=0.048 |
|        |           | Grooming time (in seconds) | Vehicle | OT | male | 12 | 36.160 (29.120, 43.280) | Mann-Whitney | p=0.723 |
|        |           |                     | Vehicle | OT | female | 11 | 28.616 (24.252, 32.172) | Mann-Whitney | p=0.723 |
|        |           | Time in zone | Vehicle | OT | male | 10 | 21.249 (19.019, 23.435) | Mann-Whitney | p=0.574 |
|        |           |                     | Vehicle | OT | female | 16 | 27.970 (21.967, 31.806) | Mann-Whitney | p=0.676 |
| 1sup3 C | EPM | Time in open-arms | Vehicle | OT | male | 21 | 38.960 (34.120,35.120) | Mann-Whitney | p=0.342 |
|        |           |                     | Vehicle | OT | female | 16 | 38.960 (34.120,35.120) | Mann-Whitney | p=0.342 |
|        |           | Open-arms entries | Vehicle | OT | male | 12 | 38.960 (34.120,35.120) | Mann-Whitney | p=0.342 |
|        |           |                     | Vehicle | OT | female | 12 | 38.960 (34.120,35.120) | Mann-Whitney | p=0.342 |
| Figure | Parameter | Brain region | Genotype | Behavioral test | Treatment | Sex | Number of individuals | Number of sections | Median (Q1,Q3) | Statistical test | p-value |
|--------|-----------|--------------|----------|----------------|-----------|-----|----------------------|------------------|---------------|----------------|---------|
| 2D     | Number of cFos positive cells | aCA2/CA3d | WT       | -SI            | -         | male | 3                    | 18               | 24.5 (19.5,28) | one-way ANOVA, Dunnett's post-hoc test | WT-SI vs WT +SI, p<0.0001 |
|        |           |              | WT       | +SI            | -         | male | 4                    | 24               | 44.5 (34.56)   | KO-SI vs KO +SI, p<0.0001 |
|        |           |              | Magel2 KO| -SI            | -         | male | 4                    | 20               | 25 (22,2.28)   |                   | n.s.    |
|        |           |              | Magel2 KO| +SI            | -         | male | 4                    | 24               | 55 (48.75)     |                   | p=0.03  |
| 2E     | Number of cFos positive cells | aDG       | WT       | -SI            | -         | male | 3                    | 18               | 39.5 (31.7,42) | one-way ANOVA, Dunnett's post-hoc test | WT-SI vs WT +SI, p<0.0001 |
|        |           |              | WT       | +SI            | -         | male | 4                    | 24               | 63 (53,79.5)   | KO-SI vs KO +SI, p<0.0001 |
|        |           |              | Magel2 KO| -SI            | -         | male | 4                    | 20               | 39 (38.46)     |                   | p=0.03  |
|        |           |              | Magel2 KO| +SI            | -         | male | 4                    | 24               | 72.5 (58.7,85.2)|                   | n.s.    |
| Figure | Parameter | Brain region | Genotype | Treatment | Sex | Number of individuals | Number of sections | Median (Q1,Q3) | Statistical test | p-value |
|--------|-----------|--------------|----------|-----------|-----|----------------------|-------------------|----------------|-----------------|---------|
| 4B     | nCi/mg of tissue equivalent | aCA2/CA3 | WT       | vehicle   | male | 3                     | 24                | 0.07469 (0.06504,0.08431) | one-way ANOVA, Bonferroni's post-hoc test | WT-vehicle vs Magel2 KO-vehicle, p<0.0001 |
|        |           |              | Magel2 KO | vehicle   | male | 3                     | 24                | 0.1395 (0.1231,0.1593) |                 | WT-vehicle vs Magel2 KO+OT, p=0.0021 |
|        |           |              | Magel2 KO | OT        | male | 3                     | 24                | 0.1254 (0.1057,0.1354) |                 | Magel2 KO-vehicle vs Magel2 KO+OT, p=0.2232 |
| 4D     | nCi/mg of tissue equivalent | vCA1/CA2/CA3 | WT       | vehicle   | male | 3                     | 24                | 0.07677 (0.06478,0.09454) | one-way ANOVA, Bonferroni's post-hoc test | WT-vehicle vs Magel2 KO-vehicle, p<0.0001 |
|        |           |              | Magel2 KO | vehicle   | male | 3                     | 24                | 0.1378 (0.1284,0.1535) |                 | WT-vehicle vs Magel2 KO+OT, p>0.9999 |
|        |           |              | Magel2 KO | OT        | male | 3                     | 24                | 0.08789 (0.06257,0.0942) |                 | Magel2 KO-vehicle vs Magel2 KO+OT, p<0.0001 |
| 4F     | nCi/mg of tissue equivalent | aDG         | WT       | vehicle   | male | 3                     | 24                | 0.1610 (0.1440,0.1953) | one-way ANOVA, Bonferroni's post-hoc test | WT-vehicle vs Magel2 KO-vehicle, p=0.0722 |
|        |           |              | Magel2 KO | vehicle   | male | 3                     | 24                | 0.1274 (0.1196,0.1415) |                 | WT-vehicle vs Magel2 KO+OT, p=0.0590 |
|        |           |              | Magel2 KO | OT        | male | 3                     | 24                | 0.1252 (0.1066,0.1517) |                 | Magel2 KO-vehicle vs Magel2 KO+OT, p>0.9999 |
Table 5

| Figure | Parameter            | Brain region | Genotype   | Treatment | Sex    | Number of individuals | Number of sections | Median (Q1,Q3)         | Statistical test     | p-value               |
|--------|----------------------|--------------|------------|-----------|--------|------------------------|--------------------|-----------------------|-----------------------|-----------------------|
| 5M     | Number of SST-positive cells | aCA2/CA3d    | WT         | -         | male   | 4                      | 65                 | 44 (33, 61)           | Mann-Whitney          | WT versus Magel2 KO p<0.0001 |
|        |                      |              | Magel2 KO  | -         | male   | 4                      | 62                 | 88 (77, 116)          |                       |                       |
| 5O     |                      |              | WT         | -         | male   | 3                      | 35                 | 43 (38, 51)           |                       | WT versus Magel2 KO+OT p<0.01 |
|        |                      |              | Magel2 KO  | OT        | male   | 5                      | 62                 | 38 (34, 45)           |                       |                       |
| 5N     | Number of SST-positive cells | aDG         | WT         | -         | male   | 4                      | 48                 | 22 (16, 29)           | Mann-Whitney          | WT versus Magel2 KO p<0.0001 |
|        |                      |              | Magel2 KO  | -         | male   | 4                      | 48                 | 48 (38, 54)           |                       |                       |
| 5P     |                      |              | WT         | -         | male   | 3                      | 35                 | 24 (21, 26)           |                       | WT versus Magel2 KO+OT p<0.0001 |
|        |                      |              | Magel2 KO  | OT        | male   | 5                      | 62                 | 18.5 (16.7, 21)       |                       |                       |
| Figure | Parameter       | Genotype | Treatment | Sex | Number of individuals | Number of neurons | Median (Q1,Q3) | Statistical test | Comparison                  | P-value |
|--------|----------------|----------|-----------|-----|-----------------------|-------------------|----------------|------------------|--------------------------|---------|
| 6C     | Glut frequency (Hz) | WT       | -         | male| 7                     | 15                | 20 (18, 35)   | One way ANOVA + Tukey post-hoc test | WT vs Magel2 KO            | n.s.    |
|        |                | Magel2 KO| -         | male| 7                     | 18                | 32 (27, 37.5) |                 | Magel2 KO vs KO+OT       | p<0.01  |
|        |                | WT       | OT        | male| 4                     | 15                | 8.5 (2.7, 16.2)|                 | WT vs WT+OT              | p<0.01  |
|        |                | Magel2 KO| OT        | male| 5                     | 21                | 11 (17.6, 23.2)|                 | WT+OT vs KO+OT           | p<0.01  |
| 6E     | GABA frequency (Hz) | WT       | -         | male| 7                     | 15                | 13.5 (2, 21)  | One way ANOVA + Tukey post-hoc test | WT vs Magel2 KO            | p<0.01  |
|        |                | Magel2 KO| -         | male| 7                     | 18                | 23.5 (21.7, 29.2)|               | Magel2 KO vs KO+OT       | p<0.01  |
|        |                | WT       | OT        | male| 4                     | 14                | 11.5 (8, 16.2) |                 | WT vs WT+OT              | p<0.01  |
|        |                | Magel2 KO| OT        | male| 5                     | 21                | 12.5 (10.5, 17.2)|               | WT+OT vs KO+OT           | n.s.    |
| 6D     | Glut amplitude (pA) | WT       | -         | male| 7                     | 15                | 42 (19, 61)   | One way ANOVA + Tukey post-hoc test | WT vs Magel2 KO            | p<0.05  |
|        |                | Magel2 KO| -         | male| 7                     | 16                | 24 (22, 34)   |                 | Magel2 KO vs KO+OT       | NS      |
|        |                | WT       | OT        | male| 4                     | 15                | 25.5 (21.7, 34)|                 | WT vs WT+OT              | p<0.01  |
|        |                | Magel2 KO| OT        | male| 5                     | 20                | 27.3 (19.3, 32)|                 | WT+OT vs KO+OT           | p<0.01  |
| 6F     | GABA amplitude (pA) | WT       | -         | male| 7                     | 15                | 29 (24.5, 33) | One way ANOVA + Tukey post-hoc test | WT vs Magel2 KO            | n.s.    |
|        |                | Magel2 KO| -         | male| 7                     | 16                | 24.5 (17.5, 37.5)|               | Magel2 KO vs KO+OT       | n.s.    |
|        |                | WT       | OT        | male| 4                     | 15                | 25 (17.4, 30.4)|                 | WT vs WT+OT              | n.s.    |
|        |                | Magel2 KO| OT        | male| 5                     | 21                | 27.1 (24, 31.5)|                 | WT+OT vs KO+OT           | n.s.    |
| Figure | Parameter                  | Genotype | Treatment | Sex   | Number of individuals | Mean ± SEM | Statistical test | p-value |
|--------|---------------------------|----------|-----------|-------|-----------------------|------------|------------------|---------|
| 6supl1 C | Glut frequency (Hz)       | WT       | male      | 5     | 13                    | 0.73 ± 0.2 | Mann-Whitney     | n.s.    |
|        |                           | Magel2 KO| male      | 5     | 16                    | 0.67 ± 0.14|                 |         |
| 6supl1 E | GABA frequency (Hz)     | WT       | male      | 5     | 12                    | 0.26 ± 0.03| Mann-Whitney     | n.s.    |
|        |                           | Magel2 KO| male      | 5     | 14                    | 23.8 ± 7.8 |                 |         |
| 6supl1 B | Glut amplitude (pA)   | WT       | male      | 5     | 14                    | 77.9 ± 2.7 | Mann-Whitney     | p<0.01  |
|        |                           | Magel2 KO| male      | 5     | 14                    | 19.9 ± 5.1 |                 |         |
| 6supl1 D | GABA amplitude (pA)   | WT       | male      | 5     | 12                    | 74.5 ± 2.7 | Mann-Whitney     | n.s.    |
|        |                           | Magel2 KO| male      | 5     | 11                    | 19.5 ± 5.1 |                 |         |

| Figure | Parameter                  | Genotype | Treatment | Sex   | Number of individuals | Median (Q1,Q3) | Statistical test | p-value |
|--------|---------------------------|----------|-----------|-------|-----------------------|----------------|------------------|---------|
| 6supl2 C | Total length apical dendrites | WT       | male      | 3     | 7                     | 3957 (3349,4372) | Mann-Whitney     | p=0.89  |
|        |                           | Magel2 KO| male      | 3     | 11                    | 3982 (3472,4780)|                 |         |
| 6supl2 D | Total length basal dendrites | WT       | male      | 3     | 7                     | 4914 (4192,5739) | Mann-Whitney     | p=0.46  |
|        |                           | Magel2 KO| male      | 3     | 11                    | 5596 (4686,6318)|                 |         |
| 6supl2 E | Mean number of apical bifurcations | WT       | male      | 3     | 7                     | 38 (20,46)    | Mann-Whitney     | p=0.31  |
|        |                           | Magel2 KO| male      | 3     | 11                    | 32 (11,37)    |                 |         |
| 6supl2 F | Mean number of basal bifurcations | WT       | male      | 3     | 7                     | 86 (46,115)   | Mann-Whitney     | p=0.25  |
|        |                           | Magel2 KO| male      | 3     | 11                    | 85 (54,125)   |                 |         |
| 6supl2 G | Mean number of apical branches | WT       | male      | 3     | 7                     | 74 (54,80)    | Mann-Whitney     | p=0.31  |
|        |                           | Magel2 KO| male      | 3     | 11                    | 64 (50,84)    |                 |         |
| 6supl2 H | Mean number of basal branches | WT       | male      | 3     | 7                     | 74 (54,80)    | Mann-Whitney     | p=0.29  |
|        |                           | Magel2 KO| male      | 3     | 11                    | 64 (50,84)    |                 |         |
| Figure | Parameter | Days in vitro | Genotype | Treatment | Number of preparations | Number of neurons | Mean ± SEM | Statistical test | p-value |
|--------|-----------|---------------|----------|-----------|------------------------|------------------|------------|------------------|---------|
| 7A     | Percentage of responsive neurons | DIV2 | WT | - | 3 | 169 | 57.88 ± 6.866 | Unpaired t test with Welch's correction | p=0.479 |
|        |           |               | Magel2 KO | - | 4 | 210 | 64.15 ± 5.519 |                       |         |
|        |           |               | WT | - | 5 | 330 | 29.42 ± 4.315 |                       | p<0.0001 |
|        |           |               | Magel2 KO | - | 3 | 175 | 62.09 ± 5.196 |                       |         |
|        |           |               | WT | - | 3 | 206 | 2.553 ± 1.052 |                       | p=0.233 |
|        |           |               | Magel2 KO | - | 2 | 179 | 1.016 ± 0.709 |                       | p=0.813 |
|        |           |               | WT | - | 2 | 92 | 1.759 ± 1.188 |                       |         |
|        |           |               | Magel2 KO | - | 2 | 97 | 1.333 ± 1.333 |                       |         |
| 7C     | Driving Force GABA (mV) | P1 | WT | - | 3 | 19 | 12.7 ± 1.5 | Unpaired t test with Welch's correction | p=0.0535 |
|        |           |               | Magel2 KO | - | 3 | 20 | 16.8 ± 1.4 |                       |         |
|        |           |               | WT | - | 6 | 42 | 5.0 ± 1.4 |                       | p=0.0358 |
|        |           |               | Magel2 KO | - | 7 | 56 | 8.9 ± 1.2 |                       |         |
|        |           |               | WT | - | 3 | 23 | 2.7 ± 1.6 |                       | p=0.6805 |
|        |           |               | Magel2 KO | - | 4 | 29 | 1.9 ± 1.1 |                       |         |
| 7D     | Driving Force GABA (mV) | P7 | WT | vehicle | 3 | 37 | 6.5 ± 1.3 | one-way ANOVA, Dunnett's post-hoc test | WT-vehicle vs WT+OT, p=0.0041 |
|        |           |               | WT | OT | 3 | 37 | 3.5 ± 1.2 |                       |         |
|        |           |               | Magel2 KO | OT | 4 | 56 | 4.1 ± 0.8 |                       | WT-vehicle vs Magel2 KO+OT, p=0.0055 |
| Figure | Parameter | Days in vitro | Genotype | Treatment | Number of preparations | Number of neurons | Mean ± SEM | Statistical test | p-value |
|--------|-----------|---------------|----------|-----------|------------------------|------------------|------------|----------------|---------|
| 7supl1 A | Amplitude of GABA-induced peaks | DIV2 | WT | - | 3 | 169 | 0.108 ± 0.007 | Unpaired t test with Welch’s correction | p=0.873 |
| | | | | | | | | | |
| | | DIV4 | WT | - | 5 | 330 | 0.101 ± 0.006 | | p=0.251 |
| | | | | | | | | | |
| | | DIV8 | WT | - | 3 | 175 | 0.112 ± 0.007 | | p=0.53 |
| | | | | | | | | | |
| | | DIV11 | WT | - | 2 | 92 | 0.047 ± 0.004 | | | |
| | | | | | | | | | |
| 7supl1 B | Amplitude of KCl-induced peaks | DIV2 | WT | - | 3 | 169 | 0.261 ± 0.011 | Unpaired t test with Welch’s correction | p=0.007 |
| | | | | | | | | | |
| | | DIV4 | WT | - | 5 | 330 | 0.309 ± 0.010 | | p=0.0059 |
| | | | | | | | | | |
| | | DIV8 | WT | - | 3 | 175 | 0.271 ± 0.010 | | p<0.0001 |
| | | | | | | | | | |
| | | DIV11 | WT | - | 2 | 92 | 0.382 ± 0.021 | | p=0.009 |
| | | | | | | | | | |
| 7supl1 C | Driving Force GABA | P7 - interneurons | WT | - | 4 | 12 | 7.9 ± 1.4 | Unpaired t test with Welch’s correction | p=0.8150 |
| | | | | | | | | | |
| | | P7 - pyramidal cells | WT | - | 6 | 42 | 5.0 ± 1.4 | | p=0.0358 |
| | | | | | | | | | |
| 7supl1 D | Resting Membrane Potential | P7 - interneurons | WT | - | 4 | 15 | -69.6 ± 2.2 | Unpaired t test with Welch’s correction | p=0.1198 |
| | | | | | | | | | |
| | | P7 - pyramidal cells | WT | - | 4 | 20 | -65.2 ± 1.6 | | p=0.4346 |
| | | | | | | | | | |
| 7supl1 E | Capacitance | P7 - interneurons | WT | - | 4 | 7 | 53.7 ± 7.0 | Unpaired t test with Welch’s correction | p=0.5908 |
| | | | | | | | | | |
| | | P7 - pyramidal cells | WT | - | 4 | 14 | 59.4 ± 7.5 | | p=0.2179 |
| | | | | | | | | | |
| 7supl1 F | Conductance | P7 - interneurons | WT | - | 4 | 12 | 12.3 ± 1.2 | Unpaired t test with Welch’s correction | p=0.8115 |
| | | | | | | | | | |
| | | P7 - pyramidal cells | WT | - | 4 | 16 | 12.6 ± 0.9 | | p=0.7526 |
| Figure | Parameter | Ratio              | Genotype | Treatment | Number of individuals (brains) | Median (Q1, Q3) | Statistical test | p-value |
|--------|-----------|--------------------|----------|-----------|-------------------------------|----------------|------------------|---------|
| 8B     | Ratio of protein quantity | KCC2/tubulin   | WT       | -         | 5                             | 5 (4.8, 5.1)   |                  | n.s.    |
|        |           |                   | Magel2 KO| -         | 6                             | 4.9 (4.5, 5)   | Mann Whitney     | p<0.05  |
|        |           | P-Ser\textsuperscript{940}/KCC2 | WT       | -         | 5                             | 0.42 (0.21, 0.54) |                  |         |
|        |           |                   | Magel2 KO| -         | 6                             | 0.16 (0.14, 0.20) |                  |         |
|        |           | P-Thr\textsuperscript{1007}/KCC2 | WT       | -         | 5                             | 0.36 (0.34, 0.66) |                  |         |
|        |           |                   | Magel2 KO| -         | 6                             | 0.49 (0.40, 0.50) |                  |         |