Characterization of the involvement of Protein Tyrosine Phosphatase H1 in neural functions

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INTRODUCTION

Protein Tyrosine Phosphatase H1 (PTPH1) is a non-transmembrane protein tyrosine phosphatase, which is regulated by a Ser/Threo switch, and characterized by an ezrin band 4.1-like domain at the N-terminus, a central PDZ domain and a catalytic domain is at the C-terminus.
PTPH1 has been demonstrated to inhibit T cell activation by dephosphorylating targets involved in TCR signaling (Gjoerloff-Wingren et al., 2000; Han S et al., 2000). In addition, it has been found to promote dephosphorilation of growth hormone receptor *in vitro* (Pasquali et al., 2003).
Little is known about the role of PTPH1 in neural functions. Sahin et al. (1995) showed that in the mouse brain, PTPH1 is expressed in thalamic areas connected to the cortex, thus suggesting a role in higher CNS functions such as learning and memory. However, to date no further evidence supporting this hypothesis has been reported.
The aim of this PhD project is to investigate the functions of PTPH1 in the CNS, using as an experimental model a genetically modified mouse lacking the catalytic domain of PTPH1. This genetic model allows the in vivo study of neural functions in the context of PTPH1 silencing . The main points addressed in the first two years of the project were (i) finding the sites of PTPH1 expression within the nervous system during embryonic development and in adult mice (ii) assessing the role of PTPH1 in CNS functions by behavioral phenotyping.

MATHERIALS AND METHODS

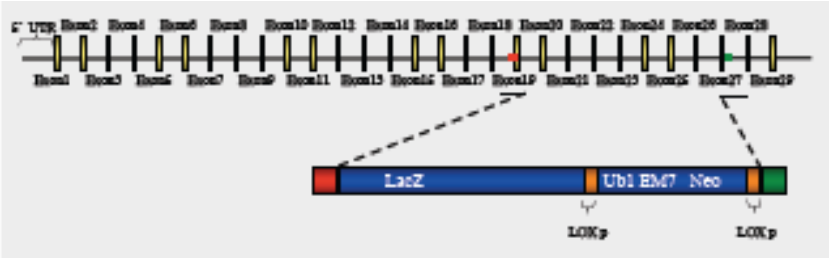
Animals

PTPH1 knockout (KO) and wild type (WT) littermates (F2 generation, 87.5% C57Bl/6 – 12.5% 129S6SvEv) aged 3-4 months were used for behavioral phenotyping. Mice were housed in single cages and maintained in a 12 hours light : 12 hours dark cycle (lights on at 7 am) at 21±1°C with food and water available *ad libitum*.
In order to study PTPH1 gene expression during development, embryos (E14 and E16) were obtained from heterozygous mating couples. Tail snips from embryos and pups were collected after death and genotyped according to the protocol reported below in “Genotyping PCR”.

PTPH1 KO design

PTPH1 KO mice were obtained from Regeneron Inc. (USA) with a proprietary Loss-of-Native-Allele procedure described by Valenzuela et al., (2003). The genomic sequence of PTPH1 from exon19 to exon27 was replaced in frame with PTPH1 initiation codon by a LacZ-Neo cassette (Fig.1). This insertion removed a genomic sequence of approximately 30KB encoding for the catalytic domain of the protein.

Fig.1: Construct of the PTP-H1 KO mouse



Genotyping PCR

Tail snips were digested overnight with proteinase K (PK, Sigma) and passed through a vacuum-column (Promega, Wizard® SV 96 Genomic DNA Purification System, cat.no. A2370) for DNA trapping. Genomic DNA was washed and eluted in water. After PK inactivation at 95°C, 2µl of DNA were used for the PCR.

Two PCR reactions were conducted in parallel (TAQ GOLD kit, 25000U, PerkinElmer N8080256). A multiple PCR reaction with three primers allowed to distinguish WT, KO and HET mice. The forward primer was designed in the flanking region of the cassette insertion site, the "reverse1" primer was designed in the deleted region and the "reverse2" primer inside the cassette (Primers sequences: forward 5'-CTGCTCTCCAGATGGAGTTG-3; reverse1 5'GCCATCTCCATCGTCACTCT-3'(for WT/HET) and reverse2 5'-CCTAGCTTCCTCACTGTTTCT-3' (for KO/HET). The couple of primers "forward/reverse1" gave an amplification product 254 bp in an agarose gel (indicating WT or HET genotypes). The couple of primers "forward/reverse2" gave an amplification product 320 bp in an agarose gel (indicating KO or HET genotype). In parallel to the multiple PCR reaction, another PCR reaction for LacZ insert was performed for confirmation, in order to distinguish HET and KO mice from WT mice (Primers sequences: Forward 5'-TCATTCTCAGTATTGTTTTGCC-3' and reverse 5'-CCACTATCAGTTGGTCACTG-3').

PTPH1 gene expression

PTPH1 gene expression in different organs was detected by LacZ staining on whole-mounts and tissue sections and by semi-quantitative RT-PCR. LacZ staining was performed also on embryo whole-mounts at different stages of development.

LacZ staining procedure was reported by Valenzuela et al.(2003). Briefly, organs and section fixed in buffered 4% PFA were incubated with a solution containing the substrate for beta-galactosidase (beta-gal, encoded by the LacZ cassette) coupled to a nitrobluetetrazolium (NBT) salt. Upon beta-gal activation the substrate and the NBT salt underwent a coupled redox reaction, which led to the precipitation of a green/blue product on the site of expression of the beta-gal. Hence, the organs in the whole-mount preparation and the tissues in the sections displayed a green/blue staining where PTPH1 gene was normally expressed.

For confirmation of LacZ staining in the brain, semiquantitative RT-PCR was performed in order to detect LacZ or PTPH1 gene expression in different brain areas of KO and WT mice, respectively. Brains from KO (N=5) and WT (N=4-5) were removed and dissected in hippocampus, cerebellum, cortex, striatum, midbrain and olfactory bulbs. RNA was extracted by Trizol Reagent (Invitrogen) and cleaned-up by an RNAeasy columns from Qiagen. 5µg of RNA were used to perform the RT-PCR reaction (SuperScript II RT kit, Invitrogen). The primers sequence for LacZ and PTPH1 amplification were the following: LacZ – forward 5'-GATGTACGTGCCCTGGAACT/reverse

5’-GGTCCCACACTTCAGCATTT; PTPH1 – forward 5’-GGGTGTGACGGAGAAGGAAT /reverse 5’-GCTCCACTCCGTAGAAGTCAA. For the amplification of a 300bp fragment of Histone2Az, the house keeping gene used as a control for equal loading of the reaction mixes, the following primers were used : H2Az forward - 5’ CGTATTCATCGACACCTGAAA; H2Az reverse - 5’ CTGTTGTCCTTTCTTCCCGAT.

Behavioral phenotyping test battery

WT and KO mice (males and females, n=10 per gender per genotype) were tested in a battery of behavioral tests for CNS and neurological functions, as reported in Fig.2.
Behavioral tests were previously pharmacologically validated and put in a sequence from the less to the most invasive. Testing sessions were performed one week apart from each other in order to allow the mice to recover.

Table 1: Experimental plan for behavioral phenotyping.

Age (wks)	8	9	10	11	12	13	14	15
Date	21-Oct	24-Oct	31-Oct	14-Nov	25-Nov	02-Dec	08-Dec	14-Dec
				15-Nov				
	arrival	quarantine	adaptation					
	10 M + 10 F							
				10 M+ 10 F	10 M+ 10 F	10 M+ 10 F	10 M+ 10 F	10 M+ 10 F
				Open field	EPM	Rotarod	Y alternation	Hot plate

The behavioral tests for neural functions were the following:

- Open field (locomotor activity and anxiety-like behavior; Ramboz et al., 1998);
- Elevated plus maze (EPM) (anxiety-related behavior; Lister, 1987);
- Rotarod (motor ability, coordination and learning; Jones and Roberts, 1968; Bontekoe et al., 2002);
- Y-maze (spatial working memory; Holcomb et al., 1999);
- Hot plate (thermal nociception; Mogil et al., 1997).

Open Field

Each mouse was placed in an open field chamber (50cm^2 wide with white floor and walls) and its locomotor activity was recorded for one hour by a video camera. A dedicated software (VideoTRACK®, ViewPoint Life Sci. Inc.) allowed to calculate the total path traveled, the percentage of time spent in the center and percentage of path traveled in the center. The total path traveled was an index of locomotor activity, whereas the time and the path in the center was an index of anxiety-like behavior.

Elevated Plus Maze (EPM)

Each mouse was placed in an apparatus consisting of four arms (29,5 cm long and 5 cm wide each). Two arms were open and two arms were delimited by black walls. The number of entries in the open and close arms was recorded by a video camera during 5 minutes and analyzed by the SMART Video-Tracking Software (..). The total number of entries into the arms was considered as an index of locomotion, whereas the percentage of time spent in the open arms and the percentage of entries in the open arms was considered ad an index of anxiety-related behaviors.

Accelerated Rotarod

The experiment was carried out on an Accelerated Rotarod apparatus for mice (Cat. # 7650 by Jones and Roberts, distr. by Basile Instr., Italy). Mice were tested for their ability to maintain a balance on a rotating bar, which accelerated from 4 to 40 rpm/min in a 5 min trial. The amount of time before the subject fell from the rod was measured. Each mouse underwent four rotarod trials (one every 30 min).

Y-maze alternation

Each mouse was placed in a Y apparatus (40 cm long /8 cm wide arms with transparent walls). The number and the sequence of the arm entries were recorded during 5 minutes. The locomotion index was calculated as the overall number of arm entries, whereas the working memory index was calculated as the number of exact alternations (entries into three different arms consecutively) divided by the possible alternations (i.e. the number of arms entered minus 2) and multiplied by 100.

Hot plate

Animals were placed on a surface heated at 52.5°C and the time interval (seconds) between placement and a shaking, licking, or tucking of the paw was recorded by the operator. The test was performed on a Hot plate apparatus for mice (Cat. # 7280 by Biol. Research Apparatus, distr. by Basile Instr., Italy) and lasted maximum 45 seconds.

Statistics

Data were analyzed by t-test or one-way ANOVA followed by Bonferroni post-hoc test. Two-way ANOVA followed by Bonferroni post-test was used to analyze the gender difference for all the significant tests.

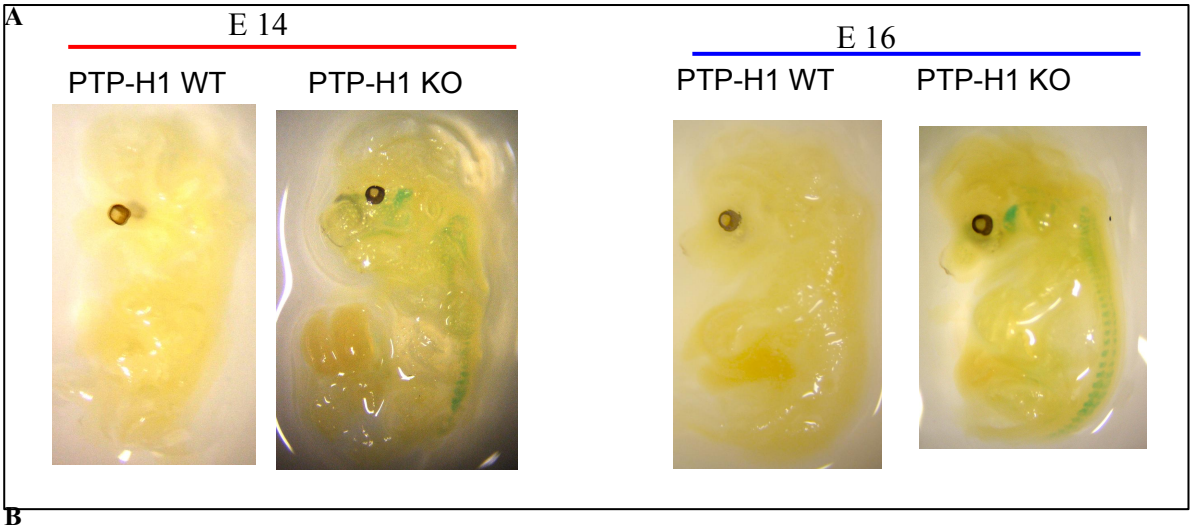
RESULTS

PTPH1 expression in the nervous system

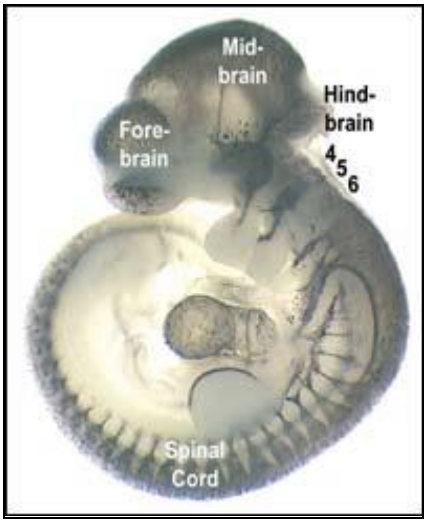
PTPH1 expression was detected at the ventral anterior brain area and the spinal cord of E14 and E16 mouse embryos (Fig.3).

Fig.3: A) LacZ staining of KO embryos at E14 and E16 in comparison with WT embryos: PTPH1 expression (indicated by blue/green staining) is localized in the brain and the spinal cord. B) Representation of CNS structures in mouse embryonic CNS. LacZ staining in KO embryos appears to be located in the ventral midbrain.

A



B



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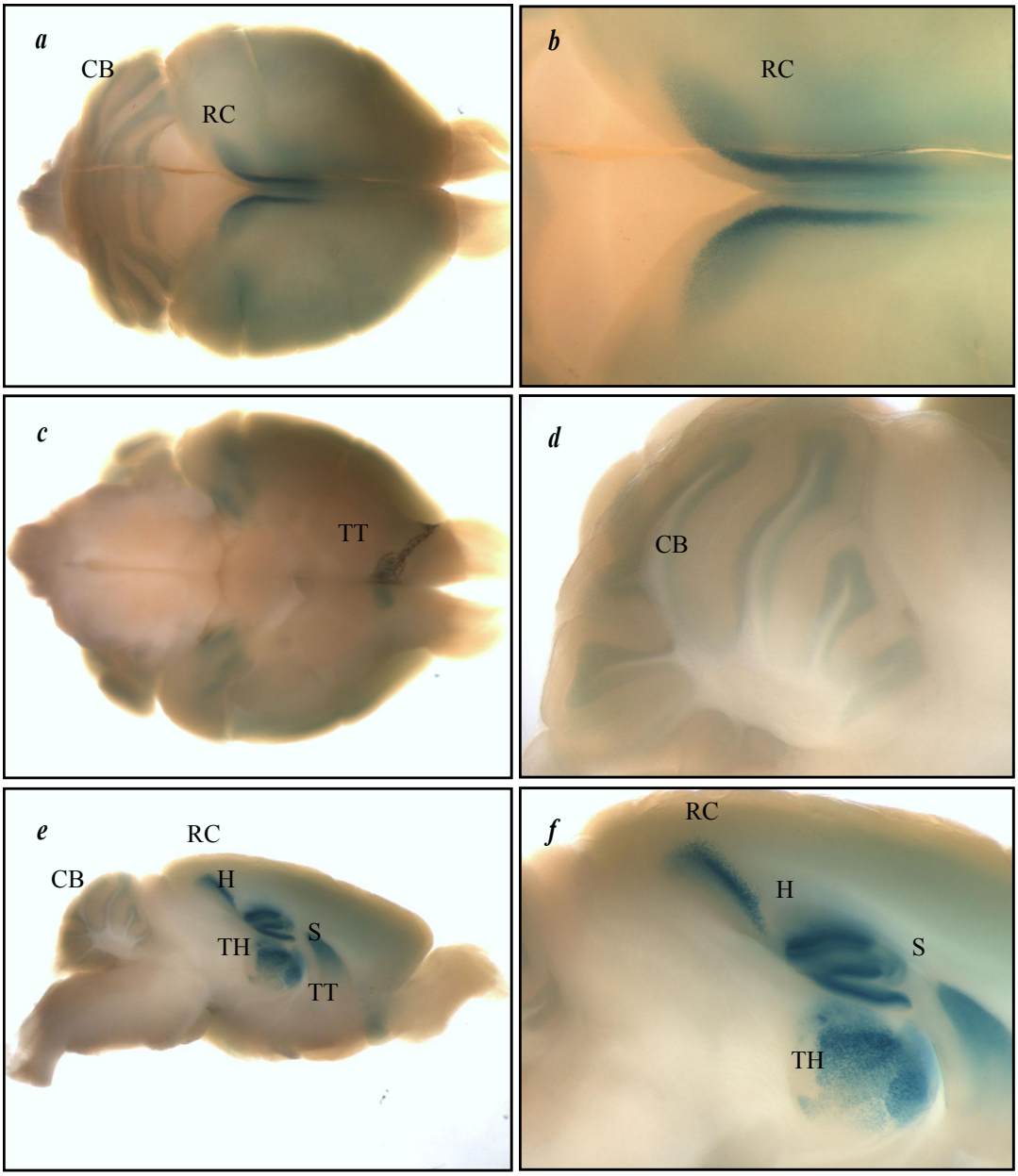
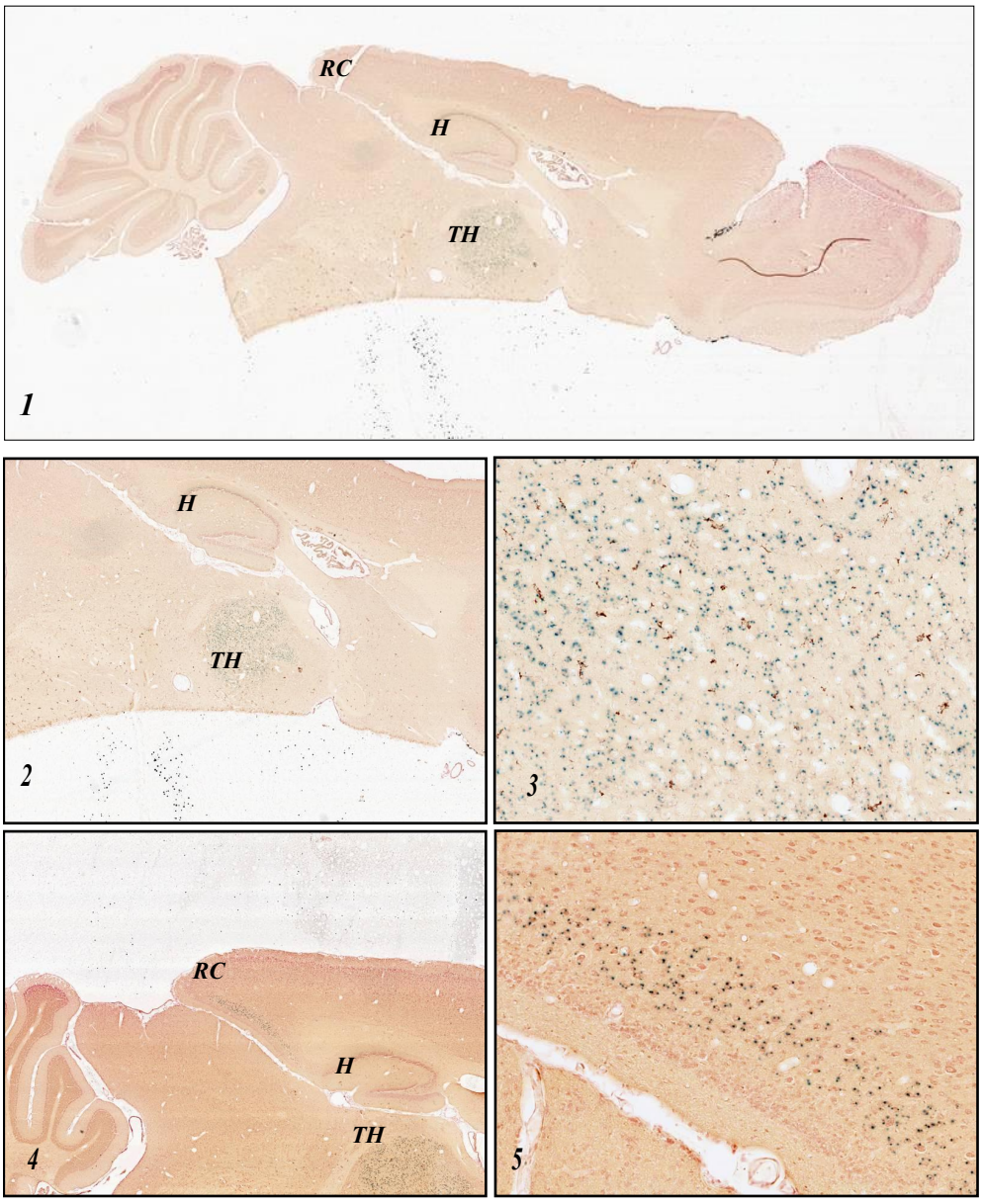
In adult brains, PTPH1 expression was detected in the cerebral and retrosplenial cortices, hippocampus, thalamus and cerebellum. LacZ staining was also present in the hypothalamus, in the area anterior to the olfactory tubercle and in the region of the Tenia Tecta (Fig.4).

Fig.4 : LacZ staining of PTP-H1 HET mouse brain. ***a:*** whole brain, dorsal view, staining in cerebellum (CB) and retrosplenial cortex (RC); ***b***: LacZ staining in the retrosplenial cortex (RC); ***c***: whole brain, ventral view, staining in Tenia Tecta (TT); ***d***: cerebellum (CB), staining in the granule cell layer; ***e, f:*** brain, longitudinal section, staining in the thalamus (TH), hippocampus (H), retrosplenial cortex (RC), septum (S), tenia tecta (TT) and cerebellum (CB).



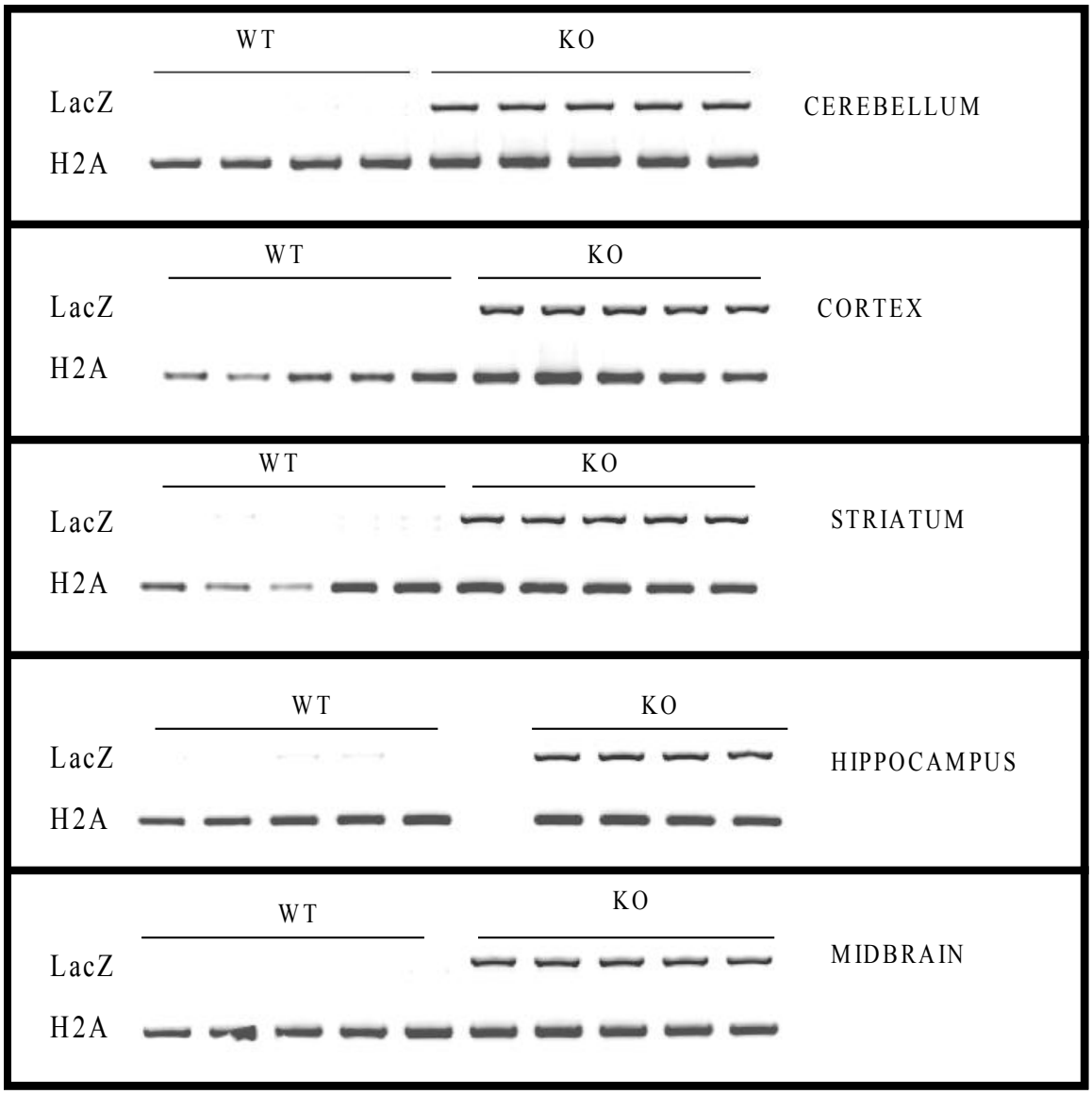
LacZ staining on sections of mouse PTPH1 HET brain confirmed the results obtained with the whole mount. PTPH1 expression was detectable in thalamus, hippocampus and retrosplenial cortex (Fig.5).

Fig.5 : LacZ staining of PTPH1 HET mouse brain. ***1:*** whole brain, longitudinal section (H, hippocampus; TH, thalamus, RC, retrosplenial cortex) ***2***: LacZ staining in the thalamus and hyppocampus; ***3***: thalamus, detail (magnification 20X); ***4***: thalamus and restrosplenial cortex; ***5:*** retrosplenial cortex, detail (magnification 13.3X)



LacZ staining has not been detected in cerebellum sections of HET mice, therefore in order to confirm the whole mount results, a semiquantitative RT-PCR for LacZ gene expression was performed on cerebellar extracts and in other brain areas, which were positively stained in both whole mounts and sections (Fig.6).

Fig.6: LacZ mRNA is expressed in brain extracts of PTPH1 KO mice but is absent in WT animals, confirming the expression of PTPH1 in the adult cerebellum, cortex, hippocampus, striatum, hippocampus and midbrain.

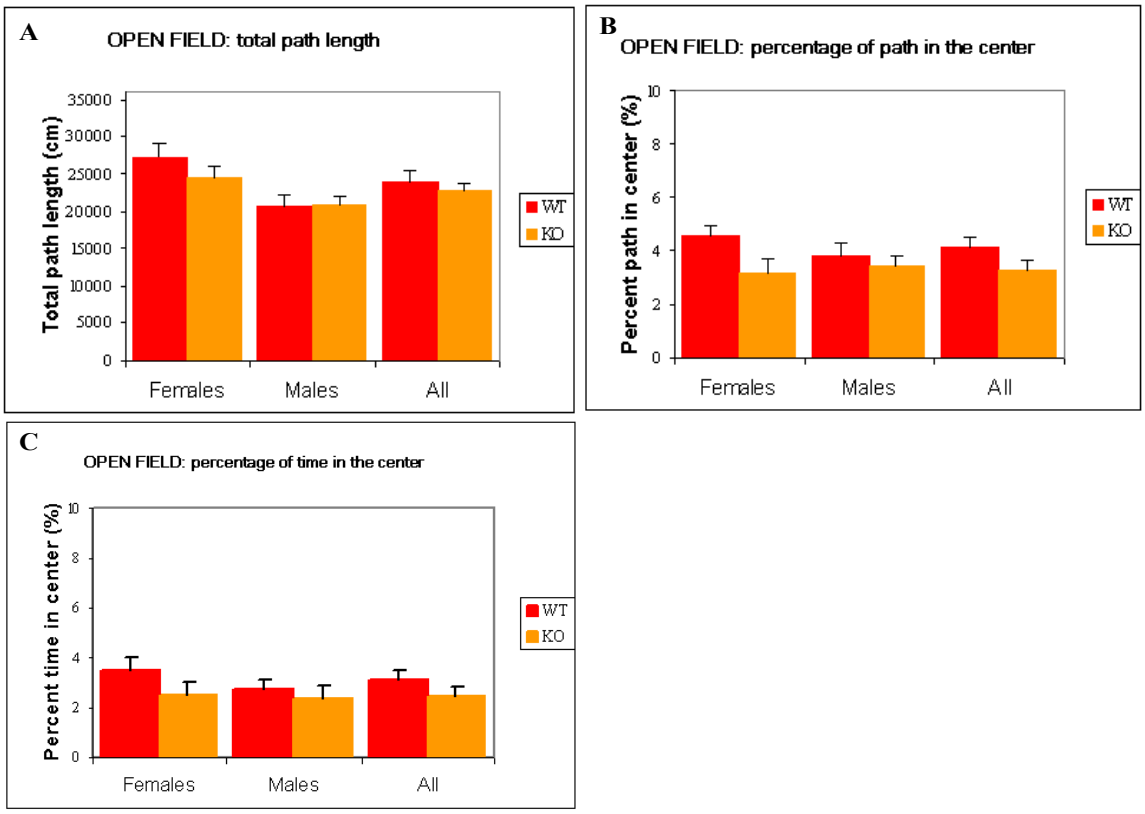


RESULTS: BEHAVIORAL PHENOTYPING

Open field

PTPH1 KO and WT mice, both males and females, displayed similar locomotor activity in the open field test, expressed as path length traveled (**Fig.7 a**; P_{male}= 0.923; P_{female}=0.246; P_{all}=0.411). Similarly, the indexes of anxiety-like behaviors showed no difference between WT and KO mice, as shown by the time spent in the center (**Fig.7 b** P_{male}= 0.618; P_{female}=0.201; P_{all}=0.179) and in the path traveled in the center (**Fig.7 c** P_{male}= 0.551; P_{female}=0.06; P_{all}=0.064).

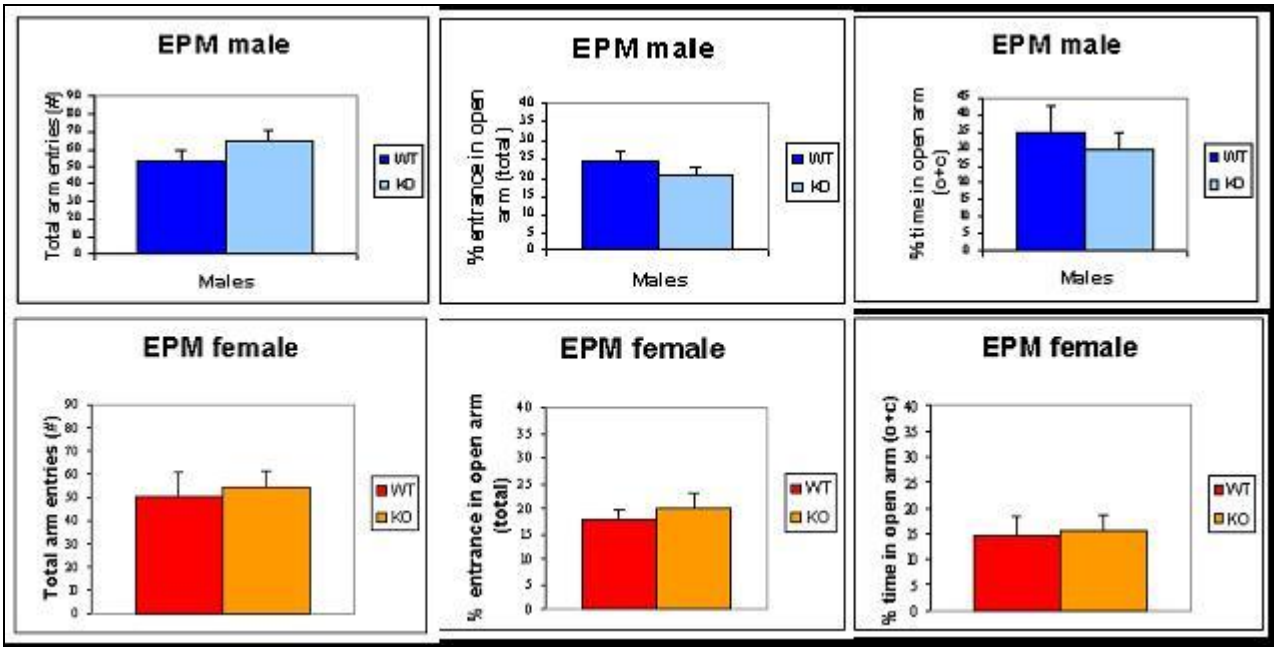
Fig.7: No differences in open field performance between PTPH1 KO and WT mice. Measurements of total path length (a), path spent in the center (b) and time spent in the center (c).



EPM

EPM test was performed for further assessment of locomotor activity and anxiety-like behavior. In line with the results of the open field test, no differences between WT and KO mice were observed in the number of arm entries (P_{male}= 0.197; P_{female}=0.759), the percentage of time spent in the open arm (P_{male}= 0.626; P_{female}=0.876) or the percentage of entrance in the open arm (P_{male}= 0.273; P_{female}=0.576) (**Fig 8**).

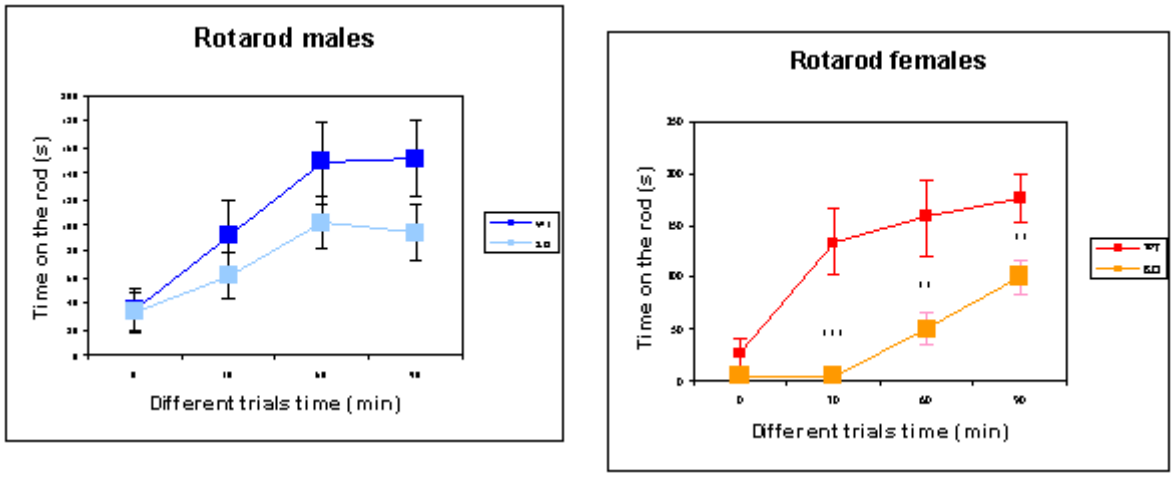
Fig.8: No effect of PTPH1 knockout on EPM performance.



Accelerating Rotarod

PTPH1 KO male mice did not show a significant difference compared to WT littermates in rotarod e performance (P_0=0.92; P_{30}=0.363; P_{60}=0.222; P_{90}=0.135) (Fig.9). Interestingly, however, PTPH1 KO female mice displayed significantly worse performance on the accelerating rotarod compared to WT littermates, starting from the second trial and onwards (P_0=0.171; P_{30}=0.002; P_{60}=0.028; P_{90}=0.025), thus suggesting not only a locomotor impairment, but also a learning deficit (Fig.9). All the groups were further analyzed by a two-way ANOVA test (genotype and gender) in order to examine whether the lower performance of female KO mice was linked to a genotype or to a gender effect . This analysis revealed a significant difference between male and female KO mice (P=0.007), thus suggesting a gender linked effect on rotarod performance.

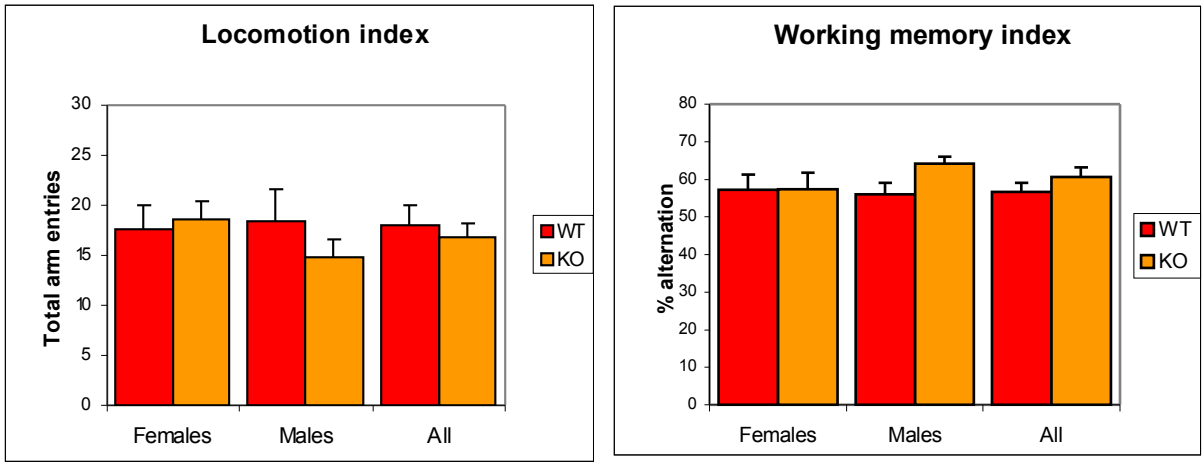
Fig.9: Significant, gender-linked impairment of rotarod performance in PTPH1 KO female mice.



Y-maze

WT and KO mice displayed similar locomotion activity in the Y-maze, indicated as total arm entries (P_{male}= 0.348; P_{female}=0.73; P_{all}=0.63) (Fig.10). However, a significantly higher working memory index (percentage of exact alteration) was found in PTPH1 KO, compared to WT male littermates (Fig. 10; P_{male}= 0.041; P_{female}=0.972; P_{all}=0.277).

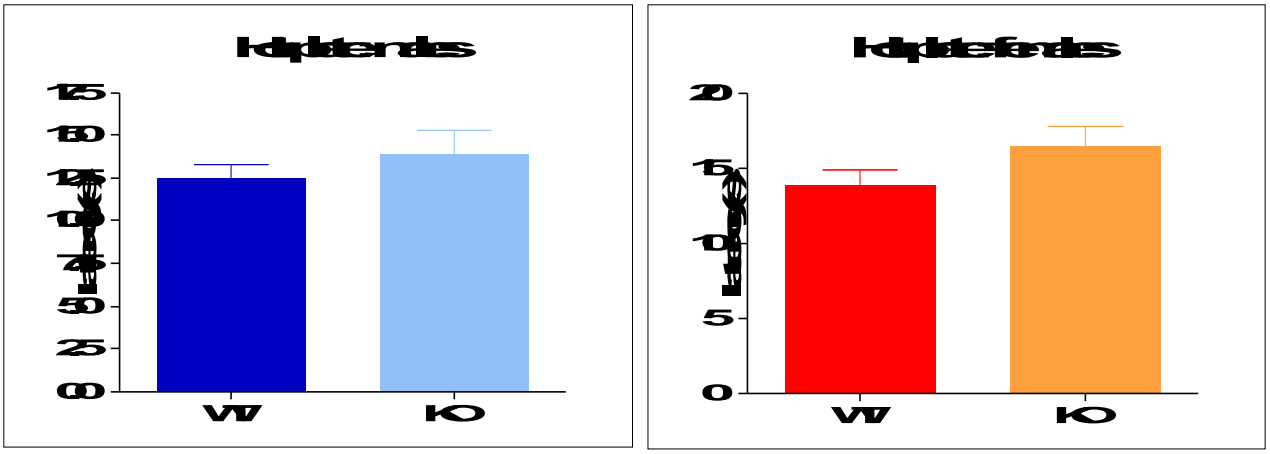
Fig.10: Similar locomotion, but enhanced working memory index in PTPH1 KO male mice.



Hot plate

No differences in thermal nociception were observed among males and females PTPH1 KO and WT mice (P_{male}=0.5288; P_{female}=0.211) (Fig.12).

Fig.12: PTPH1 KO does not affect thermal nociception.



DISCUSSION and CONCLUSIONS

Tyrosine phosphorilation/dephosphorilation has been linked to neurotransmitter release and to ion channels activity (Siegelbaum, Curr Biol 1994). Moreover PTPH1 has a high sequence homology with the members of the band 4.1 superfamily that mediate the linkage of actin filaments to the plasma membrane (Arpin et al., 1994), and therefore can be involved in cytoskeleton-membrane interactions that are crucial for axon functionality. Here we investigated the expression pattern of the tyrosine phosphatase PTPH1 in the embryonic and adult CNS, and the behavioral phenotype of PTPH1 KO male and female mice.

In adult rat brain, the pattern of PTPH1 expression includes mainly thalamic nuclei, hippocampus, pyriform cortex, neocortex and cerebellum (Sahin et al., 1995). The expression of PTPH1 in specific thalamic nuclei suggests a role for this protein tyrosine phosphatase in the transmission or in the processing of sensory information to the neocortex. PTPH1 expression in embryonic brains at E19 supports this hypothesis, being localized in the dorsal thalamic nuclei that gave origin to the thalamocortical connection (Sahin et al., 1995). Other PTPs are implicated in cellular functions, such as neurite outgrowth, that are important in CNS development (Elcheby et al., 1999; Wallace et al. 1999). Therefore, PTPH1 expression during embryonic development might be crucial for the establishment of neuronal pathways.

In order to extend the investigation on PTPH1 functions in CNS we first studied its pattern of expression in mice brain.Consistently with Sahin et al., 1995, PTPH1 expression was detected by LacZ staining in different areas, such as cerebral and retrosplenial cortices, hippocampus, thalamus, cerebellum, hypothalamus, olfactory tubercle and in the region of the Tenia Tecta. In embryonic stages E14 and E16 LacZ positive staining was found in the ventral anterior brain area and in the spinal cord. LacZ staining on sections is ongoing in order to characterize the positive brain area and to correlate it with the stained areas in adult tissues.

Subsequently, we tested PTPH1 KO mice using different behavioral assays. Interestingly, PTPH1 KO mice showed a significant difference compared to WT littermates in performance related to higher brain functions such as learning and memory, and these differences were linked to gender differences.

Particularly, PTPH1 KO female mice showed a significantly worse rotarod performance compared to WT females. Interestingly, this difference is not merely due to a coordination and motor impairment, but also to a lower learning capacity. Accordingly, some of the brain areas involved in motor coordination and learning are the cerebellum, thalamus and hippocampus, in which we have demonstrated PTPH1 expression. Moreover this analysis revealed that PTPH1 KO females are significantly impaired in rotarod performance compared to KO males. Since PTPH1 is involved in hormone signaling (Pasquali et al., 2003), the functional knocking out of the gene might have altered some neural pathways thus leading to a learning deficit.

PTPH1 KO male mice showed a higher working memory index compared to WT littermates in the Y maze test. The brain area mainly involved in working memory is the hippocampus, but the retrosplenial cortex plays an important role mostly in the spatial working memory as well (Vann et al., 2002; Mirò-Bernié et al., 2006). Accordingly, the hippocampus and retrosplenial cortex show were positive for PTPH1 expression.

In conclusion these results support the hypothesis that PTPH1 plays a role in several CNS functions, such as learning, motor coordination and working memory. However it is not clear yet through which pathways PTPH1 affects CNS functions. A deeper analysis of embryonic development in PTPH KO mice will be conducted by histological analysis and quantitive evaluation of the main neurotransmitter pathways (glutamate, acetylcholine and monoamines) in order to identify potential abnormalities in CNS architecture.

Moreover, *in vitro* experiments on mixed cortical cultures will be conducted in order to elucidate the molecular mechanisms through which PTPH1 might act (dephosphorilation of specific neurotransmitter receptors and ionic channels).

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Attended courses:

- *"Inflammatory Mechanisms in Neurodegenerative Disease"*, organized by the MS Centre ErasMS and Erasmus Postgraduate School for Molecular Medicine, held in Rotterdam, 30-31 March 2006;
- *"English course"*, organized by RBM and by the M.I.T. Center of Turin.

Attended congress:

"Neurodegenerative Diseases: Molecular Mechanisms in a Functional Genomics Framework", organized by the Max Delbruck Center for Molecular Medicine, held in Berlin, 6-9 September 2006. At this congress it was presented the poster:

PTPH1 in central nervous system: possible involvement in neurological functions.

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Attended Seminars (organized by RBM):

- *"Patologie dei roditori da laboratorio"*, held by 'Charles River Italy';
- *"Purificazione e caratterizzazione dei linfociti T soppressori dei CD8+/CD28-"*, held by P. Balestra;
- *"ATOPIC DERMATITIS: The human disease and the TNCB animal model: strengths and weakness"*, held by P.Ferro;
- *"Characterization of the Collagen Induced Arthritis (CIA) and Clinical relevant readouts"*, held by H. Asnagli;
- *"Treatments in EAE model"*, held by V. Muzio;
- *"How the brain repairs itself: new therapeutic strategies in inflammatory and degenerative CNS disorders",* held by G. Martino.

I also held a seminar in the Pharmacology Department of RBM, whose title: *"PTPH1 role in Central Nervous System"*.