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PhD THESIS WORK:

# HOMEODOMAIN INTERACTING PROTEIN KINASE 2, HIPK2, REGULATES THE EXPRESSION OF GABAergic NEURONS IN THE CEREBELLUM AND CONTROLS SHORT AND WORKING MEMORY


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# PREMISE

Homeodomain-interacting protein kinase (HIPK1-4) is a family of nuclear serine/threonine kinase, that regulating by gene transcription, affects cell proliferation, differentiation, and apoptosis (1-3). HIPK1-3 were originally described as co-repressors for homeobox transcription factors (1), in addition, they can interact with and/or phosphorylate several transcriptional regulators (4) (Figure 1).

HIPK2 is activated in response to DNA damage, including UV radiation and chemotherapeutic drugs and phosphorylates p53 to promote the transcription of pro-apoptotic p53 target genes (5-6-7). In addition, HIPK2 interacts with a number of transcription factors that control developmental processes, tumor suppression and apoptosis (4). The kinase is regulated by both sumoylation (8) and ubiquitination (9-10). Ubiquitination and subsequent degradation of HIPK2 is inhibited by DNA damaging agents. Caspase-dependent cleavage of HIPK2 removes the inhibitory domain and results in enhanced HIPK2 activity (11).

In the present study we have firstly characterized the expression profile of HIPK2 in different brain regions of adult wild type (wt) and HIPK2 Knockout (KO mice). Then, we have carried out behavioral experiments in order to establish the role of HIPK2 in several brain functions.

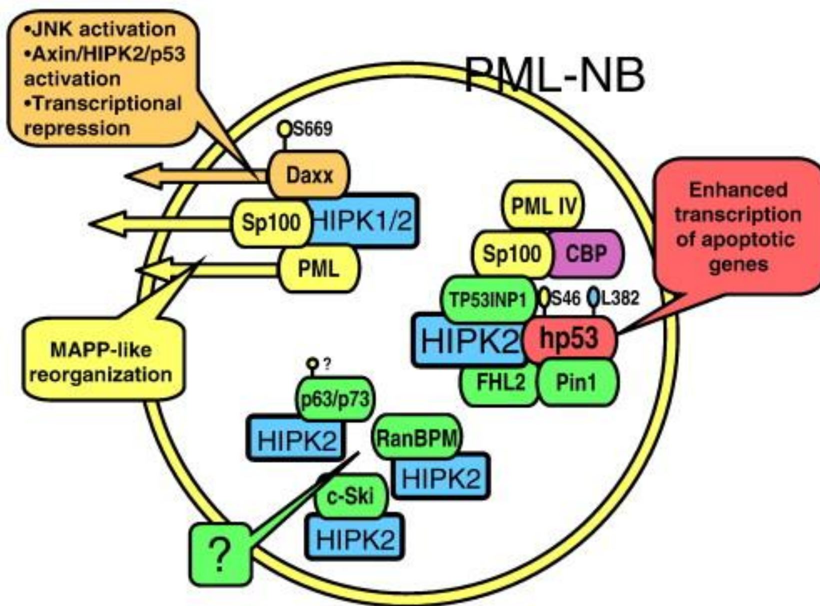


Figure 1- **Schematic representation of the interactions among HIPK2 or HIPK1 and several of their partners, which have been observed to localize to PML-NBs, at least in overexpression**. The arrows indicated the release from the PML-NBs of the indicated proteins. (Rinaldo et al. 2008)

# 1 INTRODUCTION

## 1.1 HIPK FAMILY

Homeodomain-interacting protein kinases including HIPK1, HIPK2, HIPK3 and HIPK4 (collectively designated as HIPKs) are serine/threonine kinases that constitute a family of highly conserved kinases that are involved in diverse cellular functions including cell death regulation cell survival, proliferation and differentiation (12).

Several lines of evidence now indicate that the HIPKs mediate their effects by the phosphorylation of several important proteins. For example, HIKP1, 2 and 3 have been reported to phosphorylate the homeodoamin protein NKx2.1 (13); HIPK2 phosphorylates tumor suppressor p53 on serine 46 (5-6) and HIPK3 has been reported to phosphorylate FADD, the death domain-containing adaptor protein that is critical in mediating death signals originating from the extrinsic pathway of apoptosis (2).

HIPK4 phosphorylates p53 at the level on serine 9. However the functional significance of HIPK4-mediated p53 phosphorylation remains to be fully elucidated (14).

## 1.2 STRUCTURE

HIPK2 contains 1198 amino acids and has a molecular weight of 130,965 Da. HIPK2 contains a conserved N-terminal kinase domain with a DYRK motif, a nuclear localization sequence, and a PEST domain (15).

The kinase domain of HIPK2 is followed by a protein–protein interaction domain termed Homeodomain-interacting domain (HID), which mediates interaction with homeodomain transcription factors and other molecules. The speckle retention signal

(SRS), which has been mapped next to the HID domain, appears to be essential for the characteristic subcellular localization of HIPK2 in nuclear bodies (16).

The carboxy-terminus of HIPK2 harbours an autoinhibitory domain. This domain has been identified by virtue of its interaction with Axin, an adaptor protein known from the Wnt signalling pathway, which has been shown to stimulate HIPK2 activity. Independent evidence for an autoinhibitory role of the C-terminus came from a study that demonstrated proteolytic cleavage at Asp911 and Asp977 by Caspase-6 (11-17). The end of the C-terminus of HIPK2, which harbours no known domains, is enriched in Serine/Threonine, Glutamine and Histidine residues that are frequently found in repeats (Figure 2). The functional relevance of this unusual amino acid composition remains to be elucidated (11).

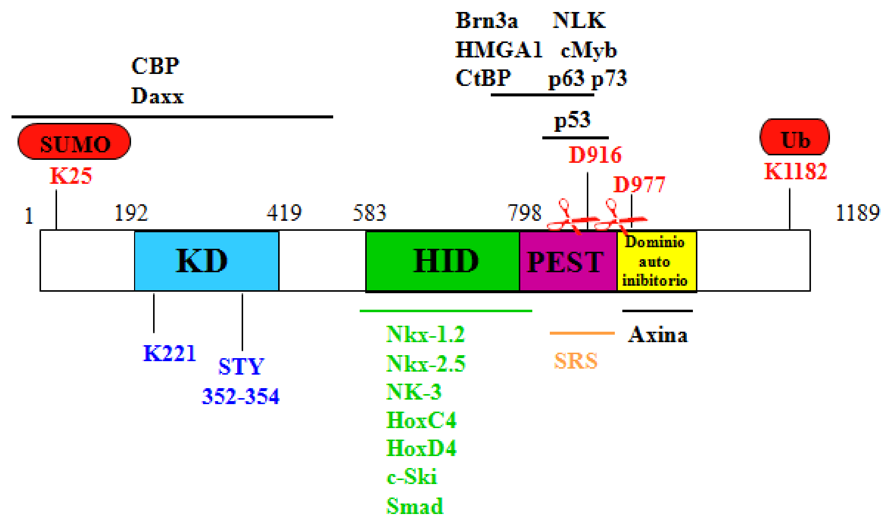


Figure 2- **Schematic representation of the murine protein HIPK2**. The horizontal lines show the regions involved in protein binding. K25: sumoylation site KD: kinase domain, inside the K221 is the lysine of the catalytic site and the STY putative regulatory site. HID: domain of interaction with homeobox factors, PEST: region containing PEST sequences; D916 D 977 E cleavage sites by caspases (indicated by scissors) SRS: the speckle retention signal; K1182: site of ubiquitination. (Rinaldo et al. 2007).

## 1.3 REGULATION

The kinase activity of HIPK2 is dependent on the presence of a lysine residue in position 221 (15).

Several research groups have demonstrated that the N-terminus of HIPK2 is subjected to post-translational modification by SUMO-1 (small ubiquitin-related modifier-1) (18). SUMO-1 is covalently linked to acceptor Lysine residue 25. Interestingly, three independent groups reported that this modification does not regulate the p53 activating function or subcellular localization of HIPK2 (18). However, SUMO modification has been found to reduce HIPK2-mediated activation of the JNK pathway and diminishes its growth inhibitory effect (18). On the other hand, SUMO modification has been shown to regulate gene expression through disruption of a HIPK2-Groucho/TLE1 corepressor complex (18). The SIM is critical for HIPK2 localization to Nuclear Bodies (NBs) and also for its recruitment to promyelocytic leukaemia (PML) NBs (Promyelocytic Leukemia Nuclear Bodies) (18).

## 1.4 DISTRIBUTION

### 1.4.1 HIPK2 distribution in embryonic and adult tissue

The 1,198-amino acid HIPK2 protein is 96% identical to the mouse protein and has a CD95-binding site between residues 754 and 899 (19). An 11.0-kb transcript is strongly expressed in neuronal tissue. A 7.8-kb transcript is also detected in uterus, and a strong 1.4-kb transcript is found in pancreas (19).

HIPK2 expression has been preferentially observed in some tissues. In fact, as shown in Figure 4, where the expression pattern at the embryonic day E16.5 is shown, HIPK2 expression occurs in the developing neural retina (NR) (Figure 3.A).

Its expression is uniform in the entire neural retina; in fact, it is present in the inner, differentiating (INL), and in the outer, proliferative (ONL), neuroblast layers (Figure 3.B). HIPK2 expression is also detectable in the telencephalon (T) (Figure 3.C); in particular, it is preferentially expressed in the ventricular proliferating zone (VZ), especially if compared to the cortical plate (CP), constituted by postmitotic cells. A high HIPK2 gene expression has been also observed in the muscle (Mu).

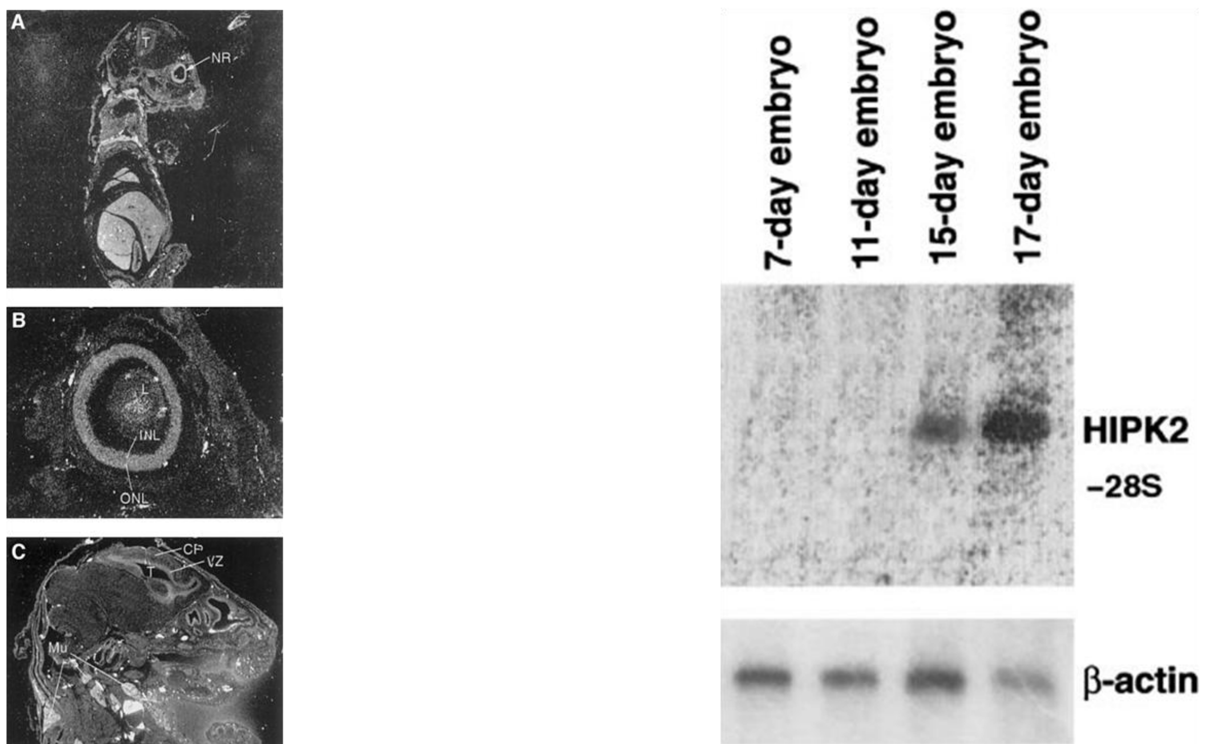


Figure3-A-B-C**HIPK2 gene expression at E16.5 post coital days in the brain.** Abbreviations are as follows: NR, neural retina; T, telencephalon; INL and ONL, the inner or the outer neuroblast layers; VZ, ventricular zone; CP, cortical plate; Mu, muscle. Expression of the HIPK2 gene in embryonic mouse tissues at different developmental stages. Poly(A)1 RNA were loaded as indicated in the figure. The panel with poli(A)1 RNA was probed with either HIPK2 cDNA or b-actin cDNA, as indicated. (Pierantoni et al. 2002).

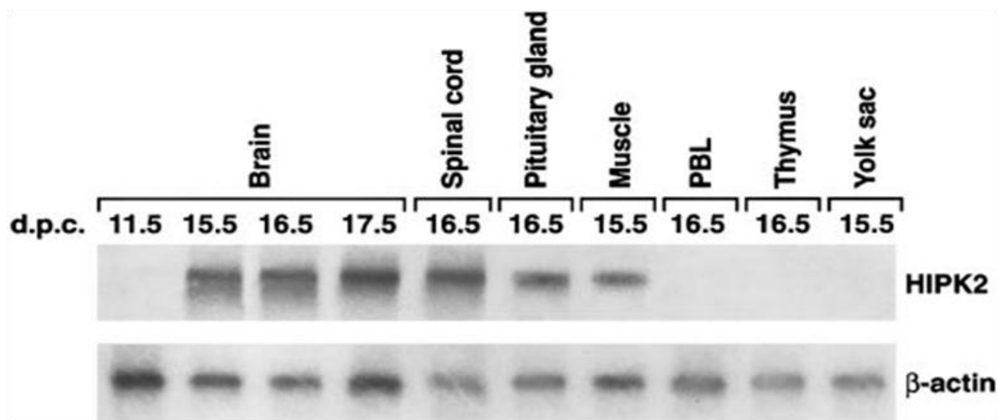


Figure4-**Analysis of the HIPK2 gene expression in embryonic tissues at different post coital days by Northern blotting**. (Pierantoni et al. 2002)

HIPK2 expression has been detected in all of the adult murine tissues. It is particularly abundant in muscle, heart, small intestine, stomach, kidney and brain. Intermediate HIPK2 gene expression levels have been observed in spleen, thymus and liver, whereas a very low level of expression has been observed in testis, skin and lung (20).

In human adult tissues, the expression levels seem to be quite low, and appear quite restricted to some of them (heart, muscle, kidney). It is almost undetectable in brain, leukocyte, testis, prostate, ovary and small intestine (20).

### 1.4.2 HIPK2 cellular distribution

HIPK2 and the highly homologous kinase HIPK3 are found in a novel subnuclear domain, the HIPK domains.

These are distinct from other subnuclear structures such as Cajal bodies and nucleoli and show only a partial colocalization with PML-NBs (Figure 1).

A kinase inactive HIPK2 point mutant is localized in the nucleoplasm. Though the architecture of HIPK domains is PML independent, HIPK2-mediated enhancement of

p53-dependent transcription, p53 serine 46 phosphorylation and the antiproliferative function of HIPK2 strictly rely on the presence of PML (21).

A recent report has shown that HIPK2 does not exclusively localize to the cell nucleus. A fraction of the kinase is also found in the cytoplasm where it associates with the actin cytoskeleton (21). The function of HIPK2 in the cytoplasm remains unknown. Elucidation of this function may reveal new roles for this enzyme, for example, in actin-dependent cell movement and migration.

## 1.5 PHYSIOLOGICAL ROLE

### 1.5.1 HIPK2 activates p53 apoptotic function in response to genotoxic stress

HIPK2 can be activated by several kinds of genotoxic stimuli, including ultraviolet radiation (UV), ionizing radiation (IR), and antitumor drugs such as cisplatin (CDDP), adriamycin (ADR) and roscovitin (6-22-23) (Figures 5-6).

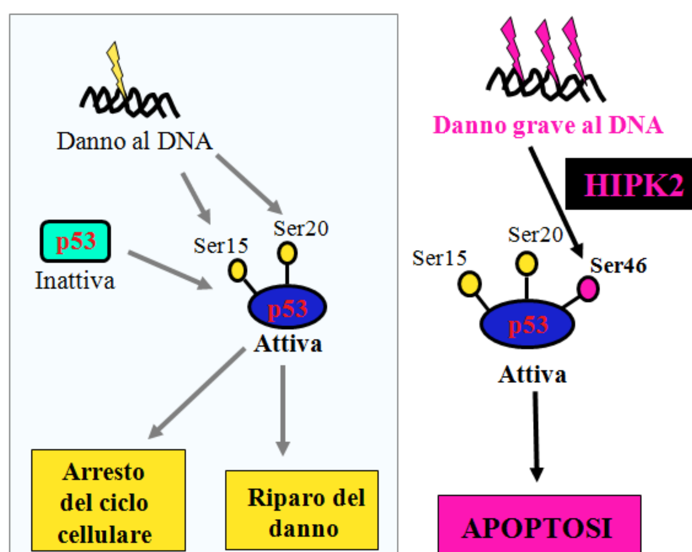


Figure 5-**Modifications and activation of p53 in response to genotoxic stress.**

One of the most studied molecules activated by HIPK2 is the oncosuppressor p53. HIPK2 phosphorylates p53 at the level of serine 46 (Ser46) and allows recruitment of histone acetylase (HAT) p300 for efficient p53 acetylation at lysine 382 (Lys382) (5). These p53 posttranslational modifications specifically induce p53-dependent pro-apoptotic gene transcription (i.e. p53AIP1, Noxa, Puma, Bax, Killer DR5) while p53 regulatory genes such as MDM2 or cell-cycle arrest related p21 are not induced (24-25). Interestingly, p53 activation induces caspase-6, which is responsible for caspase-mediated HIPK2 cleavage at positions 916 and 977 (11). This C-terminus truncated HIPK2 results in a hyperactive kinase, which potentiates p53Ser46 phosphorylation and activation of apoptosis and is eventually degraded. Thus, caspase-resistant HIPK2 mutants induce apoptosis less efficiently than wild-type counterparts (11). These findings suggest a tight regulation of HIPK2 in a p53-dependent manner, a regulatory loop similar to the elimination of ERK2 kinase by a p53-induced apoptotic program, in order to prevent ERK-mediated cell proliferation in the presence of activated p53 (26). HIPK2 knockdown impairs p53 pro-apoptotic gene transcription in response to drugs and predisposes to chemoresistance (7) and increased tumor growth in vivo (27). HIPK2 knockdown contributes to p53 inactivation by different means other than by direct impairment of p53Ser46 phosphorylation. cDNA microarray of colon cancer cells with chronic depletion of HIPK2 function by siRNA (28), showed upregulation of two novel targets of HIPK2 corepressor function that are involved in p53 deregulation, that is, Nox1 and MT2A. Thus, HIPK2 has been shown to repress Nox1 promoter activity (29). Nox1 is a homolog of the catalytic subunit of the superoxide-generating NADPH-oxidase that is often overexpressed in tumors and is involved in tumor progression and angiogenesis (30). HIPK2 knockdown induces Nox1 upregulation and Nox1

overexpression impairs p53 apoptotic transcriptional activity by inducing p53Lys382 deacetylation (29).

Interestingly, chronic HIPK2 depletion leads to p53 protein misfolding, as assessed by immunoprecipitation studies with conformation-specific p53 antibodies, that impairs p53/DNA binding and p53 transcriptional activity (28). This p53 misfolding, in colon and breast cancer cells, could be, at least in part, ascribed to metallothionein 2A (MT2A) upregulation upon HIPK2 depletion (31). Thus, MT2A depletion by siRNA, restores wtp53 native conformation and p53 function in response to drugs, in HIPK2 knockdown cells (31). Metallothionein is a family of at least 10 conserved isoforms of metal binding cysteine-rich proteins with a potential role in homeostasis of essential metals (32). MTs upregulation has been found in several human tumors including breast, colon, liver, and lung, and supports a role for MTs in acquired drug resistance (33). In most cell types, zinc is often sequestered through binding to MTs, keeping free zinc concentrations fairly low that could account for lack of function in a typical zinc-sensitive protein, such as p53 (34). Indeed, zinc supplementation to HIPK2-depleted cells restores p53 native conformation and transcriptional activity in response to drugs, as well as increases in vivo tumor regression in combination with anticancer drug adryamicin (ADR) in a xenograft colon cancer cell model (28). The finding of p53 misfolding upon HIPK2 depletion has been corroborated by in vivo studies in mice with the transgenic MMTV-neu spontaneous breast cancer model that revealed low HIPK2 gene expression in the tumor tissue compared to normal tissue, that correlates with misfolded p53 (35). Zinc treatment in combination with anticancer drug adryamicin remarkably reduces spontaneous tumor growth compared to drug treatment alone, restoring wild-type p53 (wtp53) conformation and p53 apoptotic transcriptional activity.

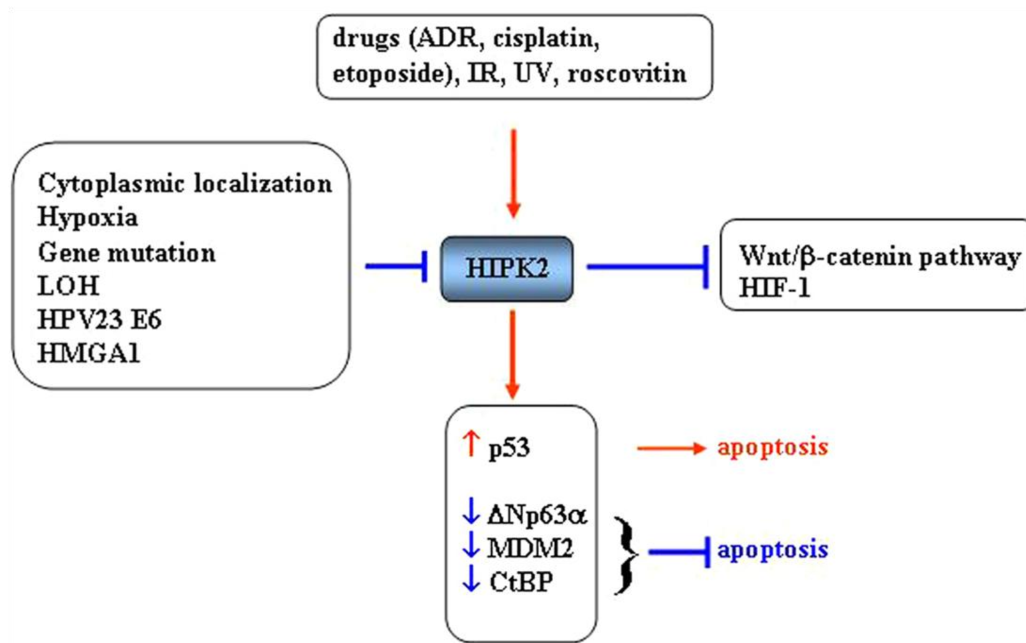


Figure 6-**Schematic representation of HIPK2 activation/inactivation**. HIPK2 can be activated by: drugs, IR, UV, roscovitin. The so far known mechanisms of HIPK2 inhibition are: cytoplasmic localization, hypoxia, gene mutation, LOH, and HPV23 E6 or HMGA1 overexpression. HIPK2 inhibits the oncogenic Wnt/β-catenin and HIF-1 pathways. HIPK2 activates p53 for apoptotic function and inhibits the anti apoptotic CtBP, MDM2 and ΔNp63α proteins. (D'Orazi et al. 2012).

### 1.5.2 HIPK2 and Wnt/b-catenin signaling pathway

The Wnt/b-catenin signaling pathway plays important roles in animal development (36). The aberrant regulation of Wnt signaling cascade component is associated with human disease, and mutations of APC, Axin and b-catenin have been studied extensively in colon and liver cancers (37). In the absence of the Wnt signal, cytosolic b-catenin is constitutively degraded via phosphorylation-dependent ubiquitination and subsequent proteasomal clearance. The Wnt ligand/receptor-induced signaling cascade results in stabilization of b-catenin and in an increase in the levels of b-catenin in the nucleus, where it functions as a coregulator of TCF/LEF transcription factors for Wnt target gene activation (38-39).

The canonical mechanism of b-catenin regulation involves a destruction complex where b-catenin is phosphorylated by priming kinases at the Ser45 site and subsequently by glycogen synthase kinase3b (GSK3b) at the Thr41, Ser37 and Ser33 sites (40-41).

HIPK2 has been shown to act as a coregulator of homeodomain transcription factors (42–43-44-45) and as a tumor suppressor through the phosphorylation of cellular target proteins, including p53, CtBP, AML and p300/CBP (46-4). HIPK2 appears to exert multiple functions depending on the binding partner or on the phosphorylation of the downstream target proteins in different signaling pathways. Although there is evidence supporting that HIPK2 may be involved in the Wnt signaling pathway (47-48-49), it is not well known how HIPK2 directly regulates intracellular b-catenin levels. Eun-A Kim et al (50) showed that HIPK2 can bind directly and phosphorylate b-catenin, and consequently degrades b-catenin. The knock-down of endogenous HIPK2 augments the stability of b-catenin and the expression of b-catenin target genes. A stable tumor cell line in which HIPK2 was silenced using an HIPK2 shRNA displays accelerated proliferation. In addition, HIPK2 expression blocked the axis duplication induced by the injection of b-catenin mRNA into Xenopus embryos (50).

These results indicate that HIPK2 is a novel negative regulator of Wnt signaling operating via the direct phosphorylation and degradation of b-catenin, a key component of the Wnt signaling pathway (50).

## 1.6 PATHOPHYSIOLOGICAL ROLE

### 1.6.1 Cancer

HIPK2 regulates cytokines is and induction of apoptosis in response to genotoxic stress, and it has been reported to act as a tumor suppressor in mice and men. Evidence from different research groups indicates that HIPK2 can suppress tumor formation through different mechanisms.

When Hipk2-/-and HIPK2+/- mice are challenged by the classical two-stage skin carcinogenesis protocol, deletion of one Hipk2 allele renders these mice more sensitive to skin cancer formation (48). These results indicate that HIPK2 acts as a haplo-insufficient tumor suppressor in mouse skin. It has been shown that patients suffering from myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML), harbour rare point mutations in the HIPK2 coding sequence that lead to the amino acid changes Arginine 868 Tryptophan and Asparagine 958 Isoleucine (51).

These point mutants localize aberrantly and show a reduced capacity to trans activate p53 target genes.

Several proteins have been shown to target the HIPK2/ p53 axis and therefore to inhibit stress- or drug-induced apoptosis to clear cancer. Recent studies demonstrated that High-mobility group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity (52). Interestingly, HMGA1 overexpression is responsible for HIPK2 cytoplasmic sequestration and the subsequent inhibition of HIPK2/p53 interaction and apoptosis activation (52).

HMGA1 is frequently overexpressed in tumors and correlates with low apoptotic index in wild-type p53 breast cancer tissues (52).

Subcutaneous injection of mice with colorectal cancer RKO cells causes tumors that grow much faster if the cells are depleted for HIPK2, indicating a growth advantage

of these cells (27). Depletion of HIPK2 results in upregulation of the integrin subunit beta4, which is strongly associated with increased migration and metastatic potential (53). Thus, it is tempting to speculate that HIPK2 might also be involved in the regulation of cell migration and metastasis formation.

Further evidence for HIPK2 being a tumor suppressor has been provided by two recent reports. In a genome-wide screen for genetic alteration in p53+/- and p53-/- mice induced by IR, more than 30% of the isolated thymic lymphomas from these mice showed a loss-of-heterozygousity (LOH) in the Hipk2 gene (54). These results suggest that Hipk2 is a potent suppressor of thymic lymphoma, and is frequently inactivated by LOH upon IR.

However, since several tumor cells maintain HIPK2 expression, although at very low levels, this suggests that residual HIPK2 expression could be of benefit, even for cancer cells.

### 1.6.2 Neurodegenerative diseases

**HIPK2 and hypoxia/ brain injury**

Oxygen deprivation (hypoxia) results in reprogrammed gene expression patterns that induce multifaceted cellular responses beside the activation of p53 pro-apoptotic function, HIPK2 exerts its antitumor activity by modulating the transcription activity of a growing number of transcription factors (55). HIPK2 binds to and represses the HIF-1α promoter (55). HIF-1α along with HIF-1β are the two subunit of HIF-1 heterodimeric transcription factor: HIF-1β is constitutively expressed in cells, while HIF-1α stability is stimulated by low oxygen (hypoxia), growth factors and several oncogenes (56-57-58). The dimer of HIF-1β and HIF-1α binds to a consensus

sequence called hypoxia-response element (HRE) and controls the expression of more than 60 genes involved in many aspects, including angiogenesis (i.e. VEGF), metabolic adaptation (i.e. GLUT1), chemoresistance (i.e. multidrug resistance 1, MDR1), invasion, and metastasis (i.e. c-MET). HIF-1α regulation includes proteasomal degradation dictated by normal or low oxygen conditions (56). Under normal oxygen tensions, HIF-1α is hydroxylated by prolyl hydroxylases, and the hydroxylated HIF-1α binds rapidly to pVHL, a negative regulator of HIF-1 that directs it for proteasomal degradation through its E3 ubiquitin ligase activity. (Figure 7)

Deprivation of oxygen by exposing cells to hypoxia or to transition metals such as cobalt ion, prevents the prolyl hydroxylase activity and stabilizes HIF-1α protein. HIPK2 binds to the HIF1α promoter along with HDAC1 within a region of active NFκB binding site that might also be involved in this regulation. The HIPK2/HDAC1 co-repressor complex induces downregulation of HIF1α expression and inhibition of HIF1 transcription activity of target genes, including VEGF. It is interesting to note that HIPK2 knockdown by siRNA switches on a genetic program that includes HIF1α and VEGF transcription allowing the development of in vitro tube formation and in vivo tumor vascularity that eventually ends up in enhanced tumor growth (55). These results strengthen the role of HIPK2 as co-repressor to hold back pathways involved in tumor development and progression.

Indeed, overexpression of HIPK2 leads to repression of HIF-1α luciferase activity and of HIF-1- dependent VEGF transcription (59). The HIPK2-induced VEGF down regulation does not only happen during hypoxia because of HIF-1 activity. An interesting pathway that regulates VEGF is dictated by β-catenin, an essential element of the Wnt signalling pathway and a potent oncogene whose accumulation has been implicated in tumorigenesis in a wide variety of human cancers, although its regulation is not yet completely understood (60-61). β-catenin acts as a

transcription factor of several target genes involved in cell growth and among them is VEGF (62). HIPK2 can suppress the β-catenin-mediated activation of VEGF transcription likely through a direct or indirect HIPK2-dependent β-catenin proteasomal degradation process (49). Nevertheless, a link between β-catenin and HIPK2 was established by a model of Hipk2 knockout mice where the β-catenin activity is a target of HIPK2 transcriptional repression, controlling cell proliferation and the propensity for tumorigenesis (48). HIPK2 is involved in both HIF-1α and VEGF regulation suggest that the loss of HIPK2 may have an enhancing effect on tumor development by acting as a “pseudohypoxic stimulus” and constitute a signal that tilts the balance toward cell survival and presumably angiogenesis growth.

Multiple lines of evidence show that the HIPK2 protein levels may be regulated by several E3 ubiquitin ligases, under normal conditions, or under some internal and/ or external stimuli according to cellular needs. Among the ubiquitin ligases that regulate HIPK2 steady-state in tumor cells by targeting it to proteasomal degradation is the WD40-repeat/SOCS box protein WSB-1,42 the RING family ligases Siah-1 and Siah-(6-10-46) and, of particular interest, the p53-target MDM2 (63). Under normal conditions, the synthesized HIPK2 is degraded constitutively by WSB-1, while under stress conditions, such as UV irradiation or cisplatin treatment, HIPK2 is phosphorylated which allows it to escape from WSB-1-dependent degradation (9). A similar regulation is dictated by Siah-1 that degrades HIPK2 in unstressed conditions, while, in response to DNA damage the HIPK2-Siah-1 complex is disrupted by the ATM/ATR-dependent phosphorylation of Siah-1 at Ser19, resulting in HIPK2 stabilization and activation (10); thus, WSB-1 is a novel predicted HIF1 target gene, as identified by an integrative genomic approach, upregulated upon hypoxia (64);

Siah-2 and HIPK2 show a mutual regulation including HIPK2-mediated Siah-2 phosphorylation that weakens mutual binding between both proteins while low

oxygen triggers increased HIPK2/Siah-2 interaction which occurs by a still unknown mechanism but that induces HIPK2 polyubiquitination (65); MDM2 is a p53 target that is activated by mild-stress and induces HIPK2 proteasome degradation (63) and that we showed being upregulated during hypoxia (66). Hypoxia-driven HIPK2 proteasomal degradation is due to p53- induced MDM2 and that HIPK2 disappearance from the nucleus becomes the mechanistic explanation of HIF-1α derepression. Moreover, as HIPK2 uses the tumor suppressor protein promyelocytic leukaemia (PML) as cofactor to efficiently phosphorylate p53 at Ser46 and as PML recruits both p53 and HIPK2 at PML nuclear bodies (NBs) (67). HIPK2 disappearance from the nucleus may affect the multiprotein complex formation and inhibit p53 phosphorylation and transcriptional activity, other than p53 proper folding, as described above. Accordingly, hypoxia-mediated HIPK2 disappearance resulted in increased p53 “mutant-like” conformation with a significant reduction of p53Ser46 phosphorylation in response to drug, and inhibition of apoptosis that can be rescued by the use of siRNA to target the hypoxia induced MDM2 (66). In summary, hypoxia can function as a mild stress that activates p53 to induce MDM2 that in turn targets HIPK2 for proteasomal degradation and inhibits p53 activity. As a proper p53 activation is crucial for drug-induced apoptosis, As a proof of principle, HIPK2 overexpression in chemical-hypoxia treated cells, results in recovery of p53Ser46 apoptotic function (59).

Another pathology involved in HIPK2 pathway is TBI.

Traumatic brain injury (TBI) is a result of an outside force causing immediate mechanical disruption of brain tissue and delayed pathogenic events that can exacerbate the injury (68).

TBI can result in neurological impairment because of immediate central nervous system (CNS) tissue disruption (primary injury) owing to mechanical force. In

addition, surviving cells may be secondarily damaged by complex mechanisms like ischemia and inflammation triggered by the primary event, leading to neuronal damage and impairments of executive function and attention (69-70-71).

HIPK2 has been consistently identified as interactors for a vast range of functional proteins, including not only transcriptional regulators and chromatin modifiers but also cytoplasmic signal transducers, transmembrane proteins, and the E2 component of SUMO ligase (72-7-74-21-7-75-76).

The transcriptional co-repressor CtBP2 (77-78-79) was recruited to the Bik promoter by the transcription factor BKLF, and ARF interacted with CtBP2 and abrogated CtBP2/BKLF repression of the Bik promoter, and promote p53-independent apoptosis (80). Unlike other transcription factors, CtBPs share a high degree of sequence homology with a group of metabolic enzymes of the 2-hA CtBP modulator identified by the yeast three-hybrid system (81) is HIPK2. In response to UV irradiation or overexpression, HIPK2 phosphorylates CtBP, targeting it for proteasomal degradation (81-82). Similar to CtBP2, HIPK2 is involved in Wnt signaling. HIPK2 in drosophila stabilizes cytosolic-catenin via phosphorylation, promoting nuclear accumulation and enhancing Wnt signaling and target gene expression (83), c-JunNH2-terminal Kinase (JNK) promotes apoptosis by phosphorylating on Ser-422 and down-regulating the transcriptional co-repressor CtBP2, inducing p53-independentapoptosis (84); however, the cross-talk between HIPK2 and JNK in CtBP2 regulation remains unknowingly. HIPK2/p53 pathway promoted neuronal apoptosis. Since CtBP2 is involved in the repression of p53-inducible pro-apoptotic genes such as Bax and Noxa, the HIPK2-mediated reduction in CtBP2 is thought to contribute to the up-regulation of Bax, Noxa, etc., and to the induction of apoptosis. HIPK2 may play pro apoptotic roles after brain injury;

however, CtBP2 present the opposite functions, promoting astrocytes activation and proliferation (84).

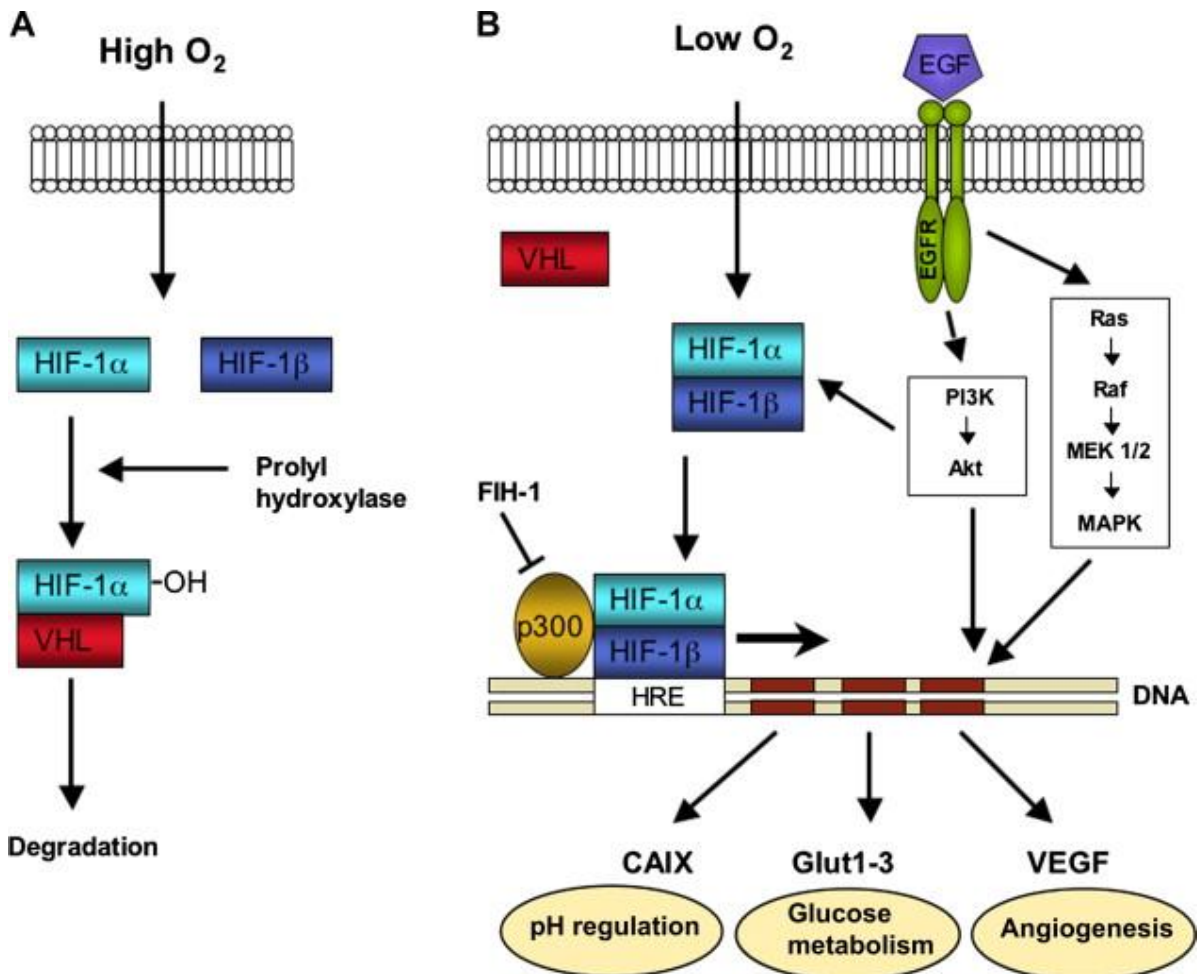


Figure 7- **Schematic representation of HIF1 pathway in normoxia (A) and hypoxia (B).** (Rademakers et al. 2008)

**HIPK2 and Parkinson's disease**

Parkinson's disease is a degenerative disorder of the central nervous system in which dopamine neurons die. Normally, these cells produce the neurotransmitter dopamine, which transmits signals along brain pathways to allow smooth,

coordinated function of the body's muscles and movement. The loss of the cells leads to progressive impairment in motor skills and speech (Figure 8).

In a study carried out using genetically engineered mice, it has been demonstrated that absence of HIPK2 caused the lack of TGFbeta3, a neurotrophin that promotes the survival of brain and nerve cells. Lack of TGFbeta3 in turn leads to the death of dopamine neurons, resulting in mice that are born with Parkinson's-like movement impairments. In summary, it has been identified HIPK2 as a transcriptional coactivator in the TGFβ signaling pathway that regulates the survival of midbrain DA neurons during programmed cell death. The results indicate that interaction with Smad3 is essential for HIPK2 function and that the loss of HIPK2 or TGFβ3 results in similar robust neuronal deficits in ventral midbrain DA neurons. These results suggest that the TGFβ-HIPK2 signaling pathway may serve as a potential therapeutic target for promoting the survival of DA neurons (85).

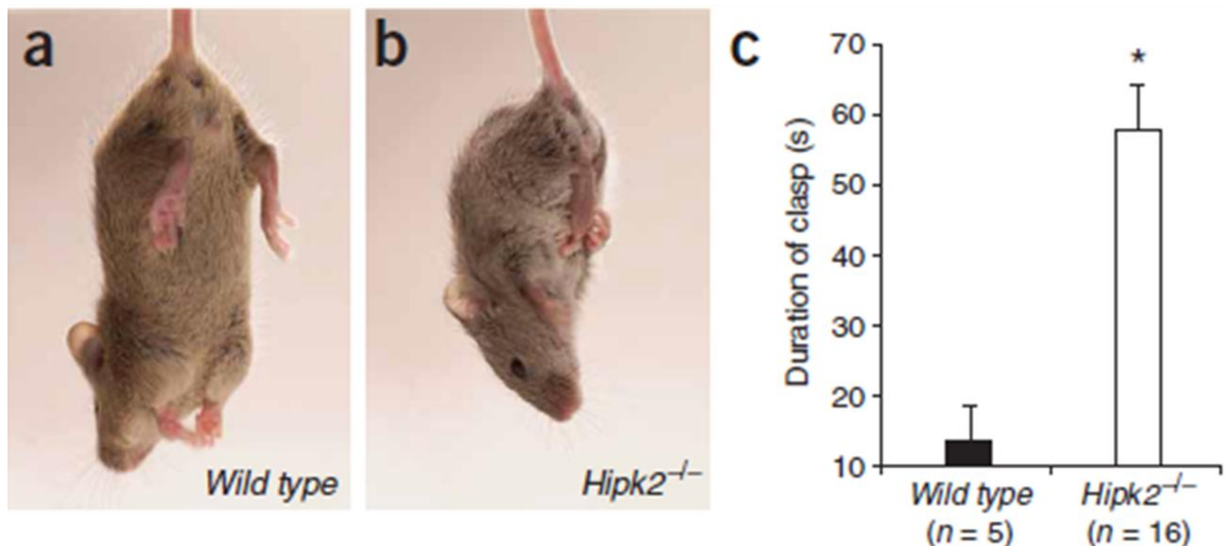


Figure 8- **Motor behavioral abnormalities in Hipk2−/− mutants**. (a–c) The majority of Hipk2−/− mutants showed hindlimb clasping when suspended by their tails. In a 2-min test, Hipk2−/− mutants spent about 60 s in the clasping position. (Zhang et al. 2006)

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**HIPK2 and Alzheimer's disease**

Alzheimer's disease (AD) is a primary progressive neurodegenerative disease where the aberrant metabolism of the amyloid precursor protein (APP) and the production and deposition of beta-amyloid peptide (Aβ) are considered responsible for neuronal death (86). An altered protein conformational state of p53, independent from point mutations, has been reported in tissues from patients with AD that led to an impaired and dysfunctional response to stressors (87-88). One of the activators that induces p53 posttranslational modification and wild-type conformational stability (24). In the absence of HIPK2, p53 acquires a misfolded conformation loosing DNA binding and transcriptional activities, depending on deregulation of metallothioneins and Zn2+ (28-31). The binding and exchange/transport of Zn2+, as well as of other heavy metals, such as cadmium or copper, are modulated by metallothionein (MT) (89). The interest in MTs derives from their role as regulators of p53 folding and activity, since small amount of MTs can induce p53 activity regulating the folding of the DBD domain through Zn2+ modulation, whereas excess of MTs reduces p53 activity by exerting their Zn2+ chelator function (90). Furthermore, an increase of MTs expression also correlates with chemoresistance, increased cell proliferation, reduced apoptosis and inhibition of p53 activity in various human tumors. The exposure to nanomolar concentrations of Aβ led to an increased content of unfolded p53 protein in fibroblasts from AD patients, compared to control subjects (91). Aβ has been hypothesized to be responsible for HIPK2 proteasomal degradation, in turn resulting in HIPK2 nuclear disappearance from target promoters such as hypoxia inducible factor 1α (HIF-1α) (55) and MT2A (31). Aβ may be responsible for important modulatory effects at cellular level before triggering the amyloidogenic cascade. One of these modulatory effects may be the inhibition of HIPK2 activity, with MT2A upregulation, in turn responsible for the induction of an altered

conformational state of p53. As a result of this conformational change, p53 loses its transcriptional activity and is unable to properly activate an apoptotic program when cells are exposed to a noxious stimulus. Altogether, Aβ-induces HIPK2 depletion and the consequent unfolded p53 may contribute to AD pathogenesis leading to dysfunctional cells (Figure 9).

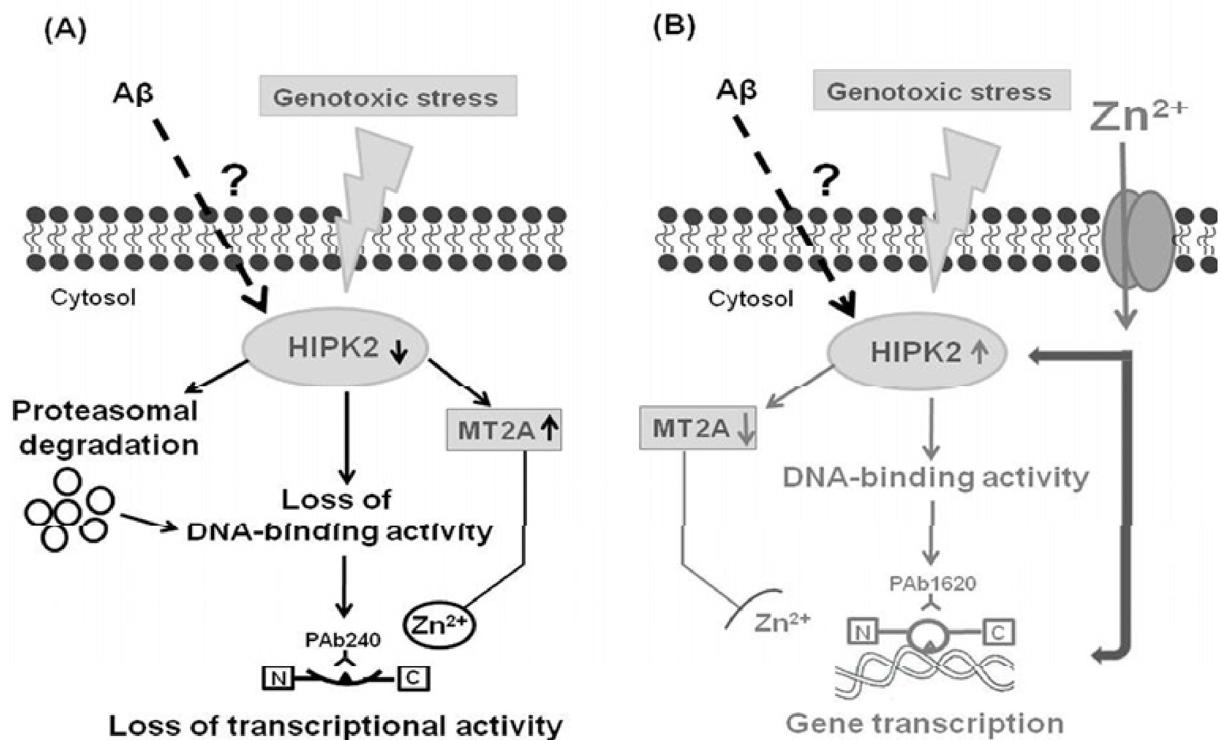



Figure 9-**Working hypothesis for a putative link among p53 protein, soluble Aβ and HIPK2.** The figure shows a novel mechanism of HIPK2 deregulation mediated by Aβ. HIPK2, when activated in response to DNA damaging agents, is able to interact physically and functionally with p53 and phosphorylate p53 at serine 46, thus regulating p53-induced apoptosis. HIPK2 also acts as transcriptional corepressor and deregulates the promote rmetallothionein 2A (MT2A). MT2A may regulate p53 activity inducing protein folding through zinc modulation. Inthe presence of soluble Aβ, HIPK2 expression and activity are inhibited through Aβ-induced degradation via the proteasome system (panel A). HIPK2 deregulation results in the induction of MT2A (panel A), that exerts its Zn2+chelator function. As a consequence, p53 protein misfolding (changing the wild-type conformation to a conformationally altered status) with subsequent abolishment of wild type p53 DNA binding and transcriptional activity occurs (panel A). Zinc supplementation counteracts Aβ effects on HIPK2 regulation (panel B). Zinc enters into cells through specific zinc transporters, that are required to convey this ion across cellular membranes, since zinc is unable to

passively diffuse across cell membranes. Zinc can directly restore p53 function (panel B). In addition, zinc can also affect HIPK2 function, thus resulting in HIPK2 reactivation (panel B). As consequence, MT2A is deregulated and p53 conformational can switch to the wild-type and transcriptional active form (panel B). (Stanga et al. 2010)

**HIPK2 and Huntigton Disease**

Huntington's disease (HD) is a devastating neurodegenerative disorder characterized by progressive and severe cognitive, psychiatric, and motor dysfunction. The disease is caused by expansion of a CAG repeat in exon 1 of IT15, which encodes for the protein huntingtin (93), with the length of the expansion correlating with age of onset and disease (94). Expression of mHtt causes increased phosphorylation of p53 on Ser46, leading to its interaction with phosphorylation-dependent prolyl isomerase Pin1 and consequent dissociation from the apoptosis inhibitor iASPP, thereby inducing the expression of apoptotic target genes. Ser46 phosphorylation and prolyl-isomerase Pin1-mediated isomerization of p53 are key events in p53-dependent apoptosis induced by mutant huntingtin (95). Interestingly, in HD brains, p53 was phosphorylated on Ser46, a modification that has been associated with activation of its apoptotic function upon stress (96). Nuclear accumulation of mHtt N-terminal fragments is observed in HD brains (97) and animal models (97). Expression of these truncated forms recapitulates many molecular and neurological HD phenotypes (98). The N-terminal fragment (residues 1–171) of either wild-type or mutant Htt (bearing 21 and 150 polyQ, respectively) were thus expressed in SH-SY5Y human neuroblastoma cells. Interestingly, mutant but not wild-type Htt induced the phosphorylation of endogenous p53 on Ser46. Alice Grison et al. (95) demonstrated that mHtt expression stimulated direct interaction of p53 with Pin1 and this effect was proportional to the amount of mHtt and inhibition of Ser46 phosphorylation by

targeting homeodomain-interacting protein kinase 2 (HIPK2), PKCδ, or ataxia telangiectasia mutated kinase, as well as inhibition of the prolylisomerase Pin1, prevents mHtt-dependent apoptosis of neuronal cells. The genetic ablation of Pin1 in HdhQ111 KI mice prevents precocious activation of p53, suggesting that Pin1 is required for induction of the p53 response, at least in early stages of HD, this implies that p53 activation might represent a general pathogenic mechanism for polyglutamine diseases sharing the occurrence of DNA lesions (95).

# 2 AIM OF THE STUDY

The mains purpose of the present thesis was to clarify the role of HIPK2 in the brain. To accomplish this aim the expression of HIPK2 was evaluated in adult mice in particular brain areas such as striatum, cortex, hippocampus and cerebellum.

Furthermore, we conducted behavior experiments on HIPK2 wild type and Knock-out. In particular, we tested the cerebellar functions; long and short term memory and finally working memory

In order to verify whether the impairments in behavioral test were associated to modification in neuronal distribution, immunofluorescence experiment have also been carried out.

# 3 METHODS

## 3.1 EXPERIMENTAL GROUPS

Sv129/c57blwt or HIPK2 KO mice were housed under diurnal lighting conditions (12hours darkness/light).

Experiments were performed according to the international guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of the “Federico II” University of Naples.

## 3.2 IMMUNOSTAINING AND IMMUNOFLUORESCENT ANALYSIS

### 3.2.1 Immunostaining and confocal immunofluorescence

Wild type and HIPK2-KO mice were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally) and perfused transcardially with 4% paraformaldehyde in phosphate buffer. The brains were sectioned coronally at 50 µm on a vibratome. The section were induced epitope retrieval at 96°C for 45 minutes in 0.01 M citrate buffer (pH 6.0). Then, the sections were incubated with PB TX 0,3% blocking solution (0,5%milk, 10%FBS, 1%BSA) for 2 hours: The primary antibody were: mouse monoclonal anti-NeuN (1:1000, Millipore), mouse monoclonal anti-MCH1 (1:200) rabbit polyclonal anti-Parvalbumin (1:500 Calbiochem), rabbit polyclonal anti-Choline Acetyltransferase (1:200 Millipore), rabbit polyclonal anti-VGluT1 (1:250 Immunological science). The sections were incubated with the corresponding fluorescent-labelled secondary antibodies (Alexa 488/Alexa 594-conjugated antimouse/antirabbitIgGs). Images were observed using a Zeiss LSM510 META/laser scanning confocal microscope. Single images were taken with an optical thickness of

0.7m and a resolution of 1024X1024. In double-labeled sections, the pattern of immunoreactivity for both antigens was identical to that seen in single-stained material. Controls of the methods in the double immunofluorescence experiments included replacement of the primary antisera with normal serum.

To control for a possible cross-reactivity between IgGs in double immunolabeling experiments, some sections were processed through the same immunocytochemical sequence except that primary antisera were replaced with normal serum, or only one primary antibody was applied, but the full complement of secondary antibodies was maintained. In addition, the secondary antibodies utilized were highly pre-adsorbed to the IgGs of numerous species. Tissue labeling without primary antibodies was also tested to exclude autofluorescence. No specific staining was observed under these control conditions, thus confirming the specificity of the immunosignals. Finally, counterstaining was performed using Mayer's hematoxylin and eosin.

### 3.2.2 Cell-counting analysis

For each strain, cell-counting of neurons was performed on a number of 3 brains harvested from at HIPK2-KO mice of 8-10 weeks of age.

For frontal cortex cell-counting experiments the brains are systematically sectioned in 8 adjacent series of slides and two series (the 1rd and the 3th) immunostained, so as to cover the whole of the frontal cortex. For cerebellum cell counting were selected 10 sections in rostro-caudal direction. The images were analyzed using ImageJ software (cell counter). The total cell number ± the standard deviations was shown

## 3.3 BEHAVIOURAL TEST

### 3.3.1 Novelty- induced exploration test

Motor activity was measured in experimental cage (30 x 25 x 30 cm) divided into central and peripheral sectors by drawn black lines. The test was performed on wild type and HIPK2-KO mice of 5,16,35 weeks of age.

Mice were placed in the arena for 30 minutes during which the distance traveled and the speed in the arena was recorded by means of dedicated software (KINOVEA free download). The data were collected when the mice were pre-symptomatic (5weeks), early symptomatic (16weeks) and fully symptomatic (35weeks).

### 3.3.2 Object recognition test

To provide mice the familiarity to the testing environment, a 2 days habituation phase was conducted by exposing each animal to the experimental cage (45 x 40 cm Plexiglas white chamber) for at least 20 minutes per day. On the training session (day 3), the box was enriched with two identical green, plastic cube objects (5x5 cm), positioned in the back left and right corners of the apparatus. Each animal was placed in the middle point of the wall freely explore and familiarize with the objects for 15 minutes. Twenty-four hours later, one of the two objects (familiar) was substituted by a new one (novel), different in color and shape (a gray, metal rectangle, 3 x 5 x 6cm). Similarly to the training procedure, each mouse was placed in the apparatus and left free to explore it for 10 minutes.

### 3.3.3 T-MAZE spontaneous alternation

In the experimental room, twenty-four hours prior to testing, mice of 5,16,35 weeks of age were familiarized to the apparatus for 10 minutes. On the following day, mice were tested in a session of ten successive free trials in the T-maze. For each trial, mice were first placed in the start box for 30 seconds (s) then allowed to explore the maze and to choose between the left and right lateral arms. After a 30-s confinement in the chosen arm, the mouse was removed and returned to the start box for the next trial. A maximum choice latency of 60 s was imposed at each trial.

### 3.3.4 Beam walking test

Sensory-motor coordination was tested using balance beams (50 cm length; 28, 11 or 5mm cross-section; 30% incline). Each mouse was given three trials per beam for three consecutive days. Latency to traverse the beam was scored and averaged. Failure to traverse the beam during the allotted time terminated the trial and the maximum time (180s) was recorded.

### 3.3.5 Wire hanging test

The wire hanging test was performed according to the method described by Niimi et al. (99) on 8 weeks old mice. The mice were held so that only their forelimbs contacted an elevated bar (50 cm in length, 2 mm diameter, 37 cm above the floor). To each mouse was given three opportunities for three consecutive days and scored as follows: 0 – fell off, 1 – hung onto the wire by two forepaws, 2 – hung onto the wire by two forepaws, but also attempted to climb onto the wire, 3 – hung onto the wire by two forepaws plus one or both hindpaws around the wire, 4 – hung onto the wire by

all four paws plus tail wrapped, and 5 – escaped. Latency to falling off was also measured up to a maximum of 60 s. Inter-trial intervals for all animals were 10 minutes.

As for all behavioral tests, apparatus was wiped with 70% ethanol between animals. The apparatus was contained in a soundproof room illuminated by a red ceiling light (80 W). A video camera above the arena was connected to a video recorder and to a monitor located in an adjacent room.

### 3.4 RNA EXTRACTION AND QUANTITATIVE RT-PCR (qRT-PCR)

Total RNA was extracted from tissues and cell cultures with Trizol (Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

qRT-PCR analysis was performed by using Quantitect Reverse trascription kit (QIAGEN, Hilden, Germany). One microgram of total RNA was reverse transcribed using an optimize dbl end of oligo-dT and random primers according to the manufacturer's instructions. PCR was carried out on cDNA as previously described. To design a quantitative reverse transcription-PCR (qRT-PCR) assay, we used the Human Probe Library system (Exiqon). qRT-PCR for Hipk2 was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the following primers:

mHipk2forward:CCACATGTCAATTGCCTCAC

mHipk2 reverse: AGGTCATCGACTTTGGTTCAG

The 2___Ct formula was used to calculate the differential gene expression.

### 3.5 STATISTICAL ANALYSIS

Values are expressed as ± S.E.M. Statistical analysis was performed with 2-Way ANOVA, followed by Newman-Keuls test.

Behavioral test data were analyzed using the nonparametric Kruskal-wallis test. Statistical significance was accepted at the 95% confidence level ($P<0,05$)

# 4 RESULTS

## 4.1 HIPK2 shows a brain region specific pattern of expression.

Real time PCR analysis revealed that HIPK2 gene expression, reached the maximum value in cerebellum of 1month and 3 months, and decreased with the age. (Fig.10).

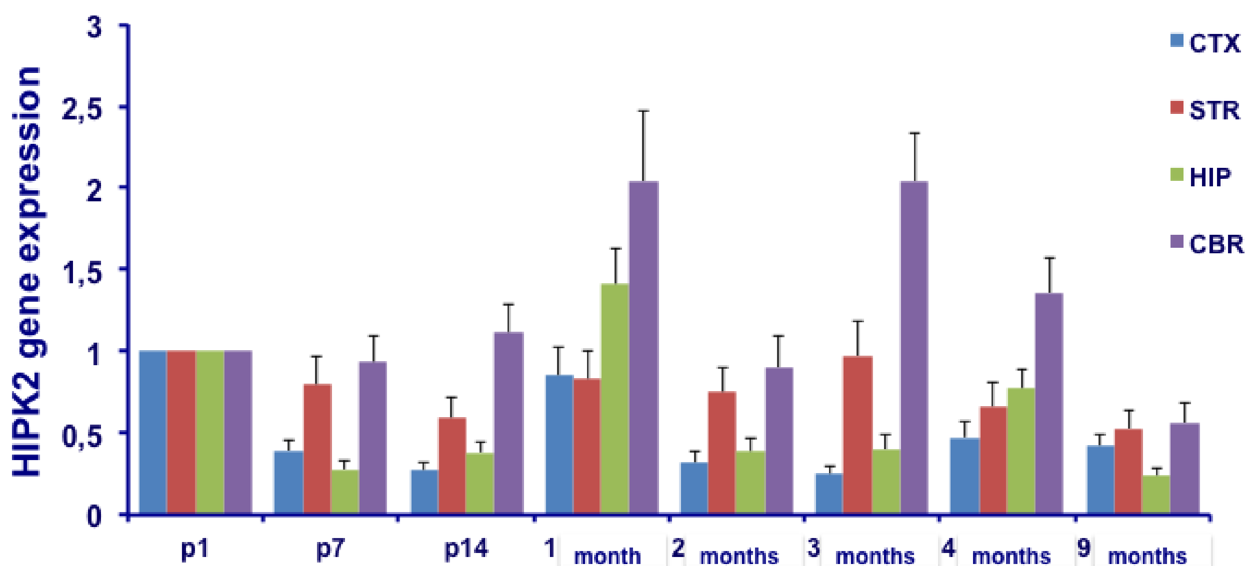



Fig 10-**HIPK2 gene expression in fold change** revealed that the expression of HIPK2 gene was constant and the maximum level of mRNA was in cerebellum. The value of HIPK2 for each sample was normalized to the corresponding value of G6PDH expression

In wild type animals the single label immunohistochemical study showed the maximum expression of HIPK2 in most neuronal perikarya of the cerebral cortex, hippocampus, caudate putamen, and cerebellum.(Figures 11-12-13-14)

HIPK2 immunoreactive in striatum displayed a nuclear and cytoplasmic localization (Figure 14) while in the cortex, hippocampus and cerebellum the localization appeared to be mainly cytoplasmic (Figures 13-12-11).

In particular, as concern the brain cortex, HIPK2 was expressed in primary (M1), secondary (M2) motor cortex and cingulate cortex (CG) while in the remaining area of the cortex it appeared less expressed (Figure 13).

As concerning the hippocampus, HIPK2 immunoreactivity was expressed exclusively in CA2 and CA3 regions, whereas it seemed not expressed in CA1 and dentate gyrus (Figure 12).

Finally, in cerebellar cortex, the pattern of HIPK2 expression was very interesting as it appeared over expressed in all Purkinje cells, as identified by Calbindin and Parvalbumin antibody whereas, it appeared less expressed in deep stellate cells (basket cells) and outer stellate cells of molecular layer, as visualized with Parvalbumin antibody (Figure 11).

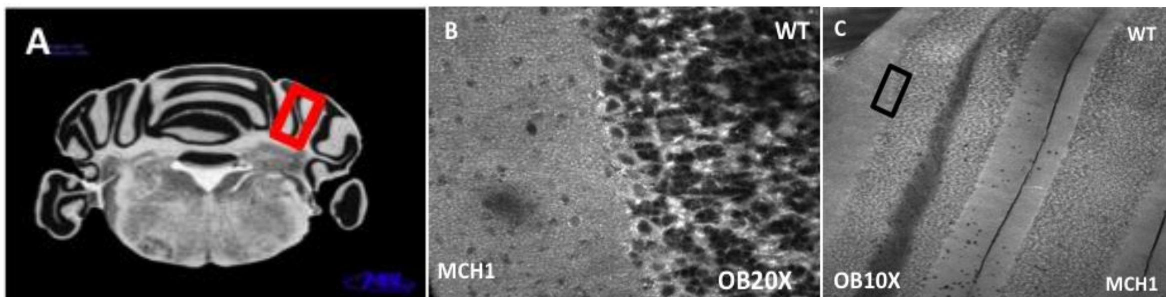



Figure11-**HIPK2 distribution in cerebellar cortex.** HIPK2 immunohistochemistry-processed sections from wt mice at 8-10 weeks of age in cerebellar cortex. A representative image of cerebellum Bregma: -5.84 mm. B- MCH1 (HIPK2) expression in the Purkinge layer and granular cells of cerebellar cortex. Ob.20x. C- MCH1 (HIPK2) expression in cerebellar cortex.Ob.10x.

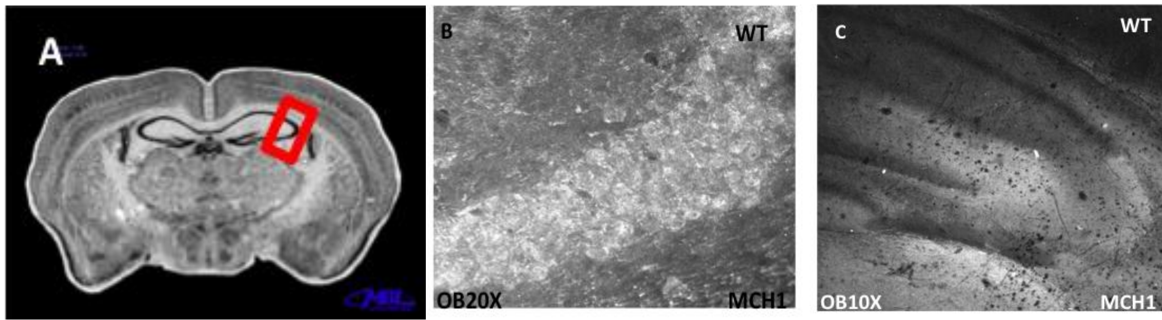



Figure12-**HIPK2 distribution in hippocampus.** HIPK2 immunohistochemistry-processed sections from wt mice at 8-10 weeks of age in hippocampus. A- representative image of hippocampus. Bregma: -1.06 mm. B- MCH1 (HIPK2) expression in the CA2-CA3 hippocampal area. Ob.20x. C- MCH1 (HIPK2) expression in hippocampus.Ob.10x.

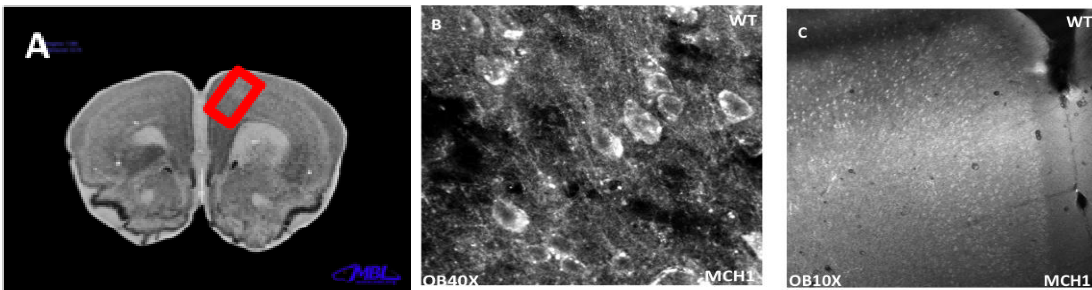



Figure13-**HIPK2 distribution in cerebral cortex.** HIPK2 immunohistochemistry-processed sections from wt mice at 8-10 weeks of age in cerebral cortex. A- representative image of cerebral cortex. Bregma: 1.70 mm B- MCH1 (HIPK2) expression in M2-M1 motor cortex. Ob.40x. C- MCH1 (HIPK2) expression in motor cortex and cingulate cortex. Ob.10x.

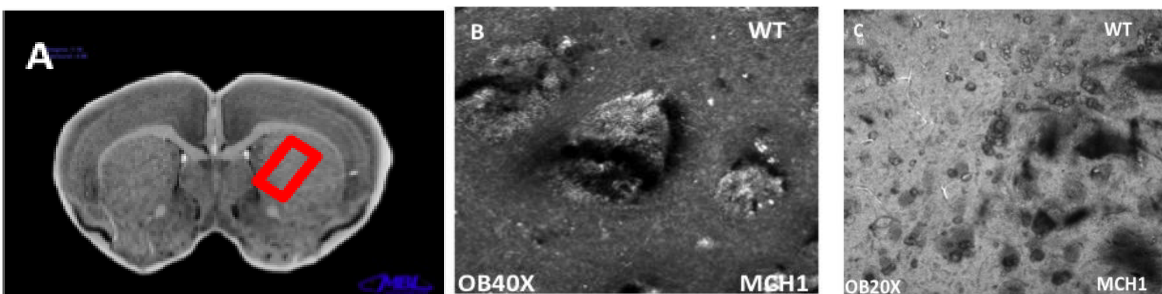



Fig 14-**HIPK2 distribution in caudate putamen.** HIPK2 immunohistochemistry-processed sections from wt mice at 8-10 weeks of age in striatum. A- representative image of striatum Bregma: 1.18 mmB- MCH1 (HIPK2) expression in striatum. Ob.40x. Zoom 2 C- MCH1 (HIPK2) expression in caudate putamen. Ob.20x.

## 4.2 HIPK2 is expressed in all medium spiny neurons and parvalbuminergic inter-neurons

In order to characterize the kind of neurons expressing HIPK2 double label immunohistochemical studies have been carried out.

The results of these experiments showed that HIPK2 immunoreactivity was distributed to the perikarya of all parvalbuminergic (PV) inter-neurons (Figures 15-16) and medium spiny neurons (CALB) as identified respectively by Parvalbumin and Calbindin immunoreactions. HIPK2 immunoreactivity was almost absent in the glutamatergic neurons and cholinergic inter-neurons as identified by VGLUT1 and CHAT antibodies.

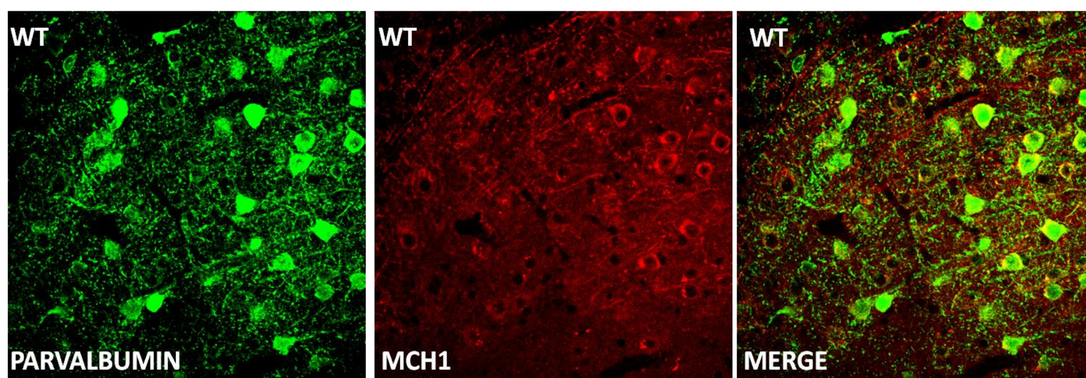



Figure 15-**HIPK2 and Parvalbumin interneurons labeling in cerebral cortex of wt animals.** Confocal laser scanning microscopy (CLSM) images of double-label immunofluorescence for MCH1 (HIPK2) (red) and interneurons marker parvalbumin (green). Ob.40x. Zoom 0.7.

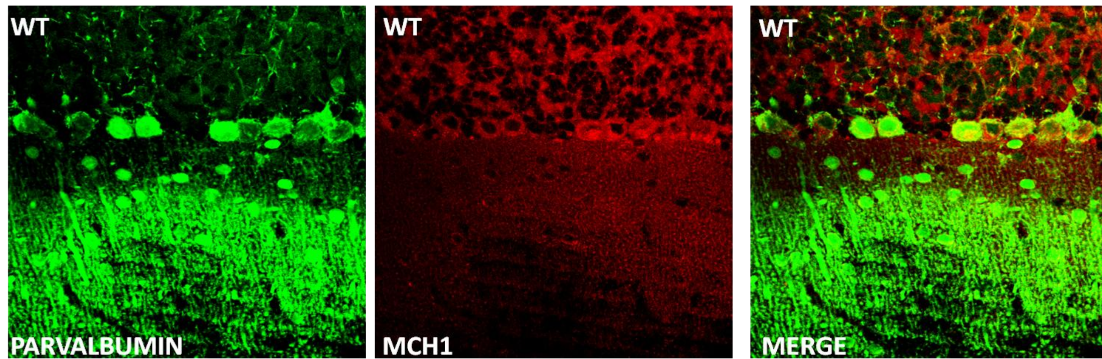



Figure 16-**HIPK2 and Parvalbumin interneurons labeling in cerebellar cortex of wt animals.** Confocal laser scanning microscopy (CLSM) images of double-label immunofluorescence for MCH1 (HIPK2) (red) and interneurons marker parvalbumin (green). Ob.40x. Zoom 0.7.

### 4.3 The lack of HIPK2 is associated with a reduction in cerebellar GABAergic neurons

In the HIPK2 KO animals the single label immunohistochemical study direct against NeuN (Neuronal Nuclei) showed a significative reduction of neurons present M2 frontal cortex (Figure 17).

This alteration appeared to be as a large specific lesion located exclusively in M2 region and involved a specific neuronal population, represented by the Parvalbuminergic inter-neurons (Figure 19). In fact, in M2 frontal cortex of HIPK2 KO mice, a dramatic 43% reduction in PV positive inter-neurons has been observed in comparison to WT animals (Figure 18).

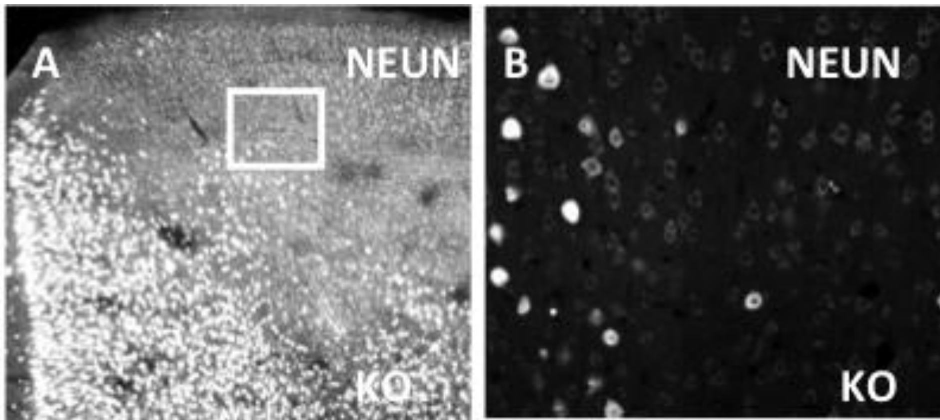



Figure 17-**NeuN immunohistochemistry-processed sections from HIPK2-KO mice in cerebral cortex.** A- NeuN labeling in HIPK2-KO mice.Ob.20x. B- NeuN labeling in HIPK2-KO mice Ob.40x.

That the effect is region specific has been demonstrated by the lack of significant differences in the number of PV positive neurons of M1 and cingulate cortex of HIPK2 KO mice compared to the wt animals.

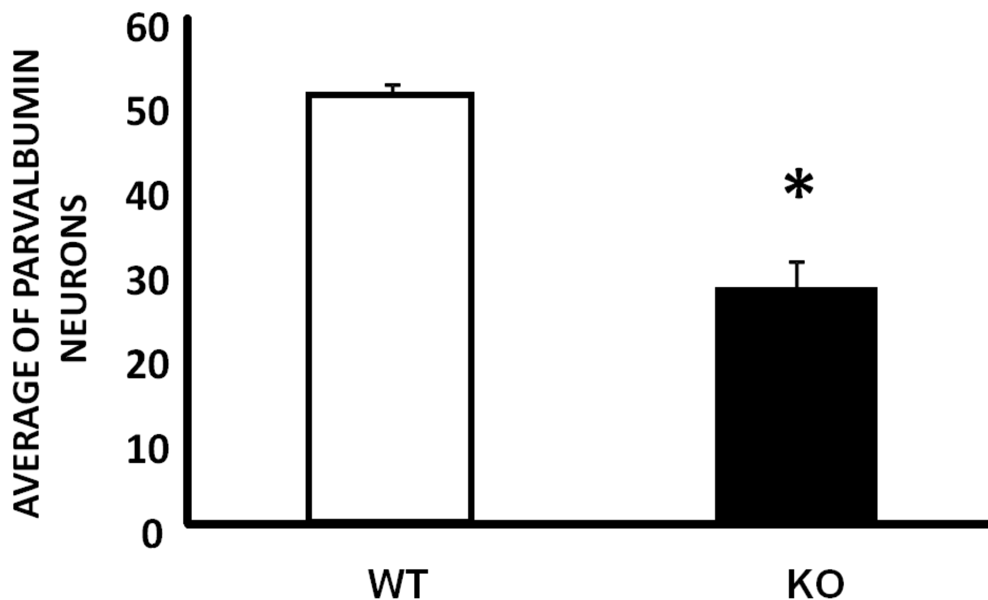



Figure 18-**Average of parvalbumin neurons in M1/M2 motor cortex**. Cell counting analysis on a number of 3 brains for each strain between 8-10 weeks of age. The number of parvalbumin positive cells is lower in HIPK2 KO mice compared to wt animals

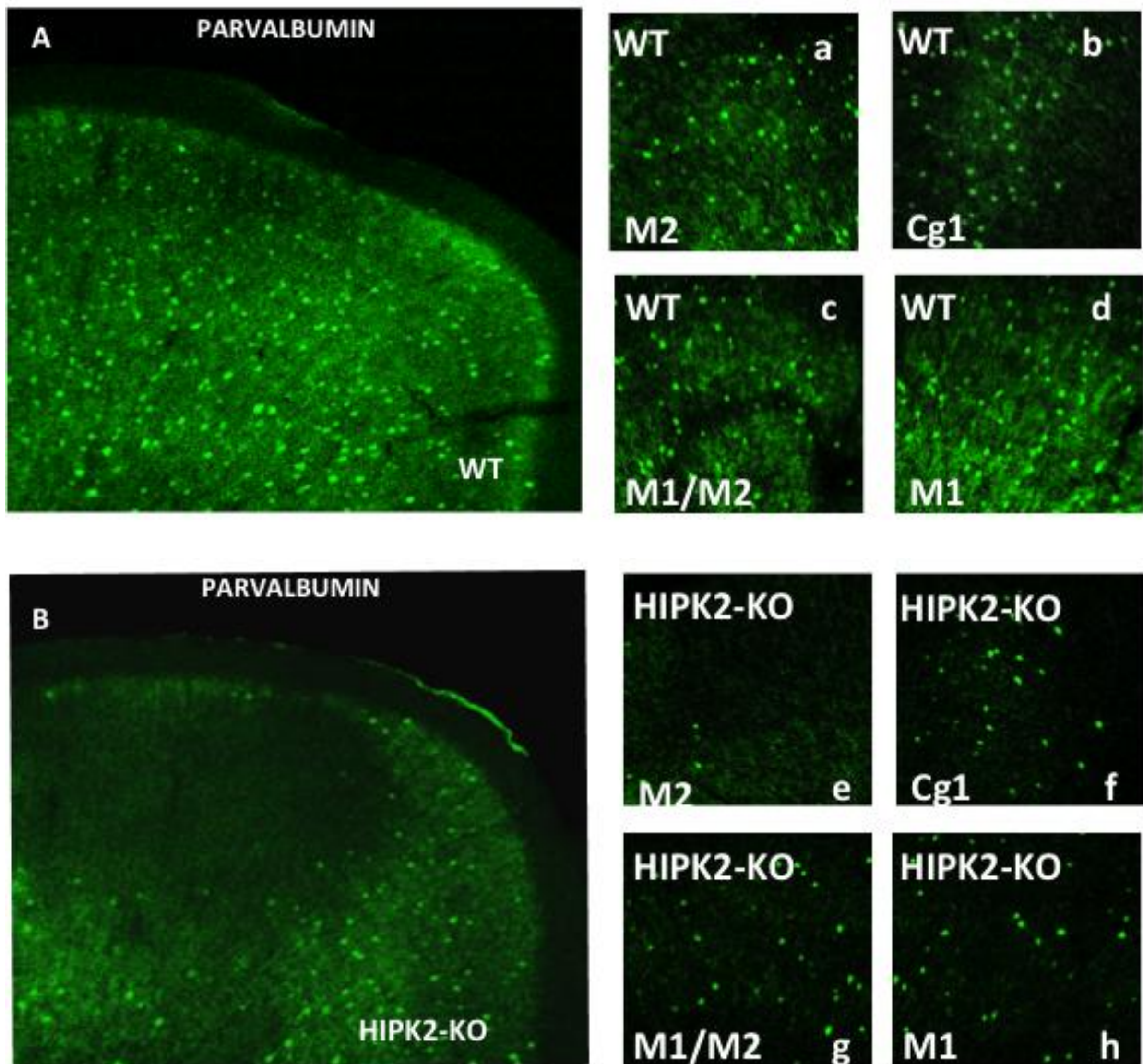



Figure 19- **Parvalbumin neurons distribution in frontal cortex.** Confocal laser scanning microscopy (CLSM) images of single-label immunofluorescence for parvalbuminin cerebral cortex of wt and HIPK2-KO mice. A- Parvalbumin expression in cerebral cortex of wt mice. Ob.10x. a- parvalbumin neurons (pv+) in secondary motor cortex M2. b- pv+ in cingulate cortex. c- pv+ in secondary and primary motor cortex. d- pv+ in primary motor cortex. B- parvalbumin neurons in cerebral cortex of HIPK2-KO mice Ob.10x. e- pv+ in secondary motor cortex. f- pv+ in congulate cortex. g- pv+ in secondary and primary motor cortex. h- pv+ in primary motor cortex. a-b-c-d-e-f-g-h Ob.20x.

Interestingly, the number of Calbindin positive neurons in M2, M1 and cingulated cortex of HIPK2 KO animals did not change.

In the hippocampus, no significative variations in the number of Parvalbumin and Calbindin positive neurons has been observed.

Interestedly, although in striatum of HIPK2 KO mice no change in the number of Calbindin and Parvalbumin positive cells has been detected, the cellular localization of HIPK2 appeared to be completely different from the cytoplasmic localization observed in hippocampus and cortex.

Indeed HIPK2 appeared to be present in the cytoplasm and in the nucleus, of all parvalbumin positive cells present in the striatum.

The most dramatic effect due to the lack of HIPK2 has been observed in the cerebellum. Indeed, in the cerebellar cortex of HIPK2 KO mice it has been detected a severe 60% loss of Calbindin (Figures 20-21) and Parvalbumin positive inter-neurons in Purkinge cell layer (PCs) (Figures 22-23).

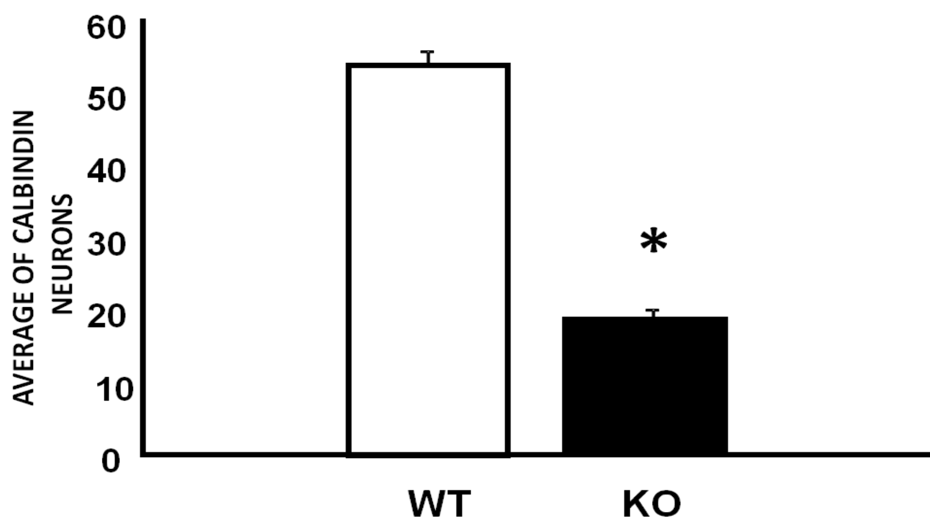



Figure 20- **Average of medium spiny neurons in cerebellar cortex**. Cell counting analysis on a number of 3 brains for each strain between 8-10 weeks of age. The number of calbindin positive cells is lower in HIPK2 KO mice compared to wt animals.

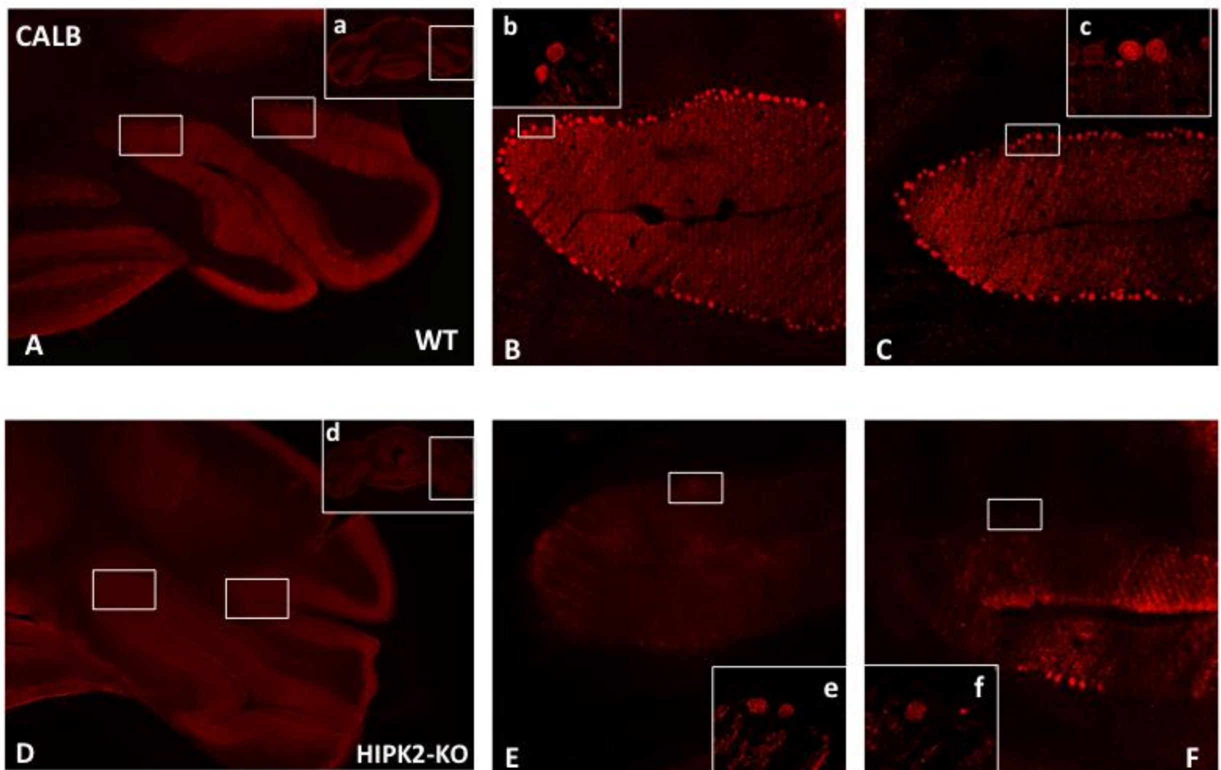



Figure 21-**Calbindin neurons distribution in cerebellar cortex of wt and HIPK2-KO mouse.** Confocal laser scanning microscopy (CLSM) images of single-label immunofluorescence for calbindin of wt and HIPK2-KO mice. A- Calbindin labeling in cerellar cortex of wt mice.Ob.4x. a- Low magnification. Ob. 2x. B-C Calbindin labeling in cerellar cortex of wt mouse Ob.20x. b-c high magnification Ob.100x.D- Calbindin labeling in cerellar cortex of HIPK2-KO mouse. Ob.4x.d- Low magnification. Ob.2x. E-F Calbindin labeling in cerellar cortex of HIPK2-KO mouse Ob.20x. e-f high magnification Ob.100x.

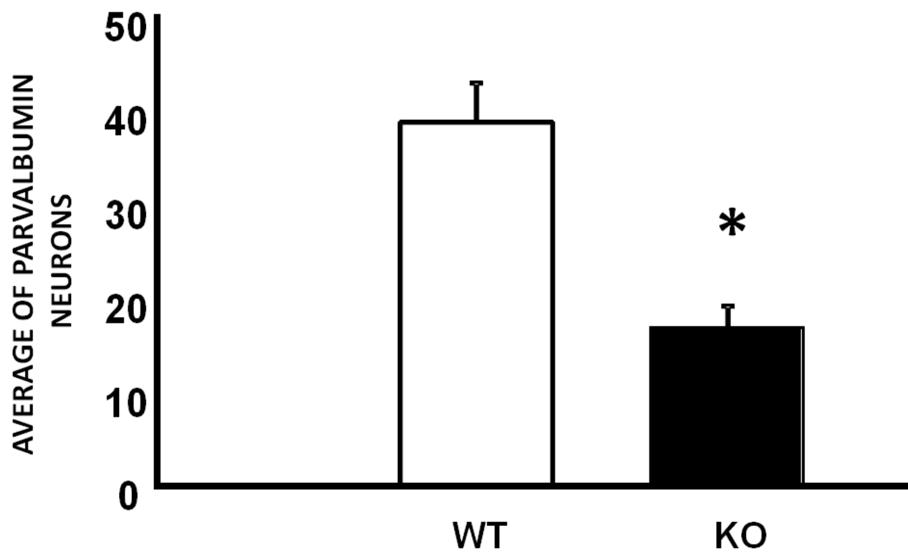



Figure 22-**Average of parvalbumin neurons in cerebellar cortex**. Cell counting analysis on a number of 3 brains for each strain between 8-10 weeks of age. The number of parvalbumin positive cells is lower in HIPK2 KO mice compared to wt animals.

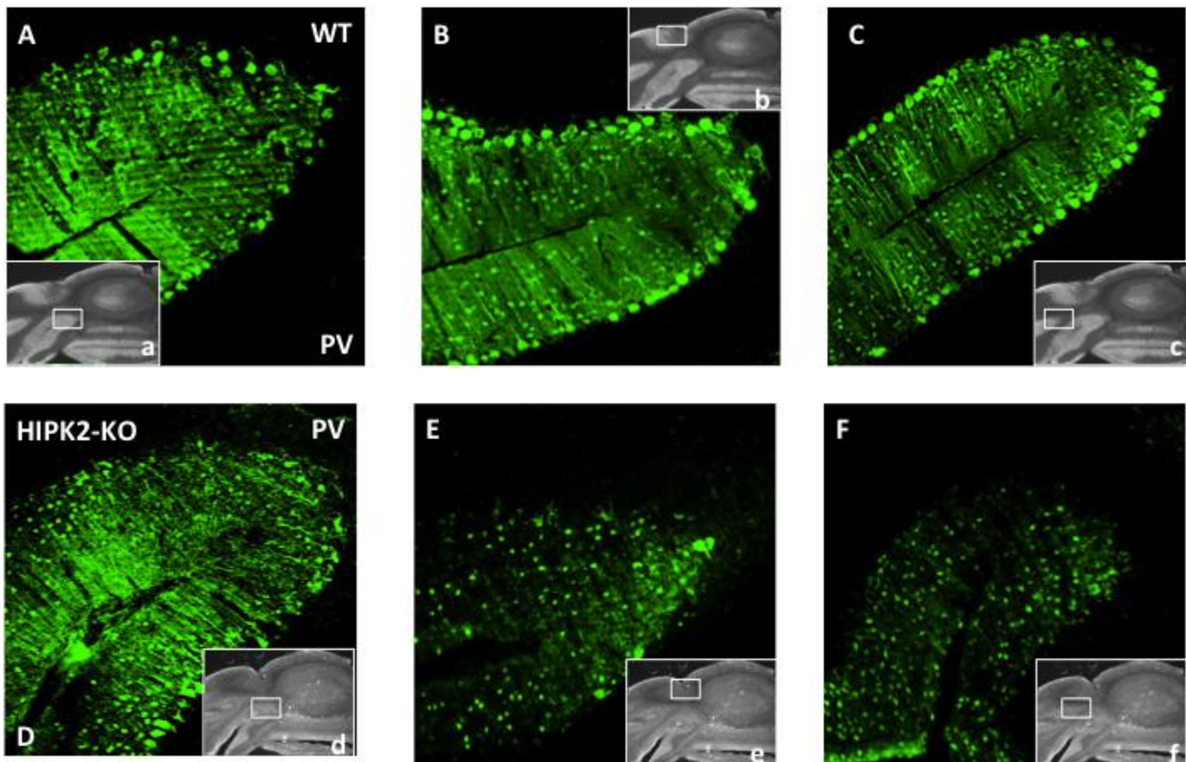



Figure 23-**Parvalbumin neurons distribution in cerebellar cortex of wt and HIPK2-KO mouse.** Confocal laser scanning microscopy (CLSM) images of single-label immunofluorescence for parvalbumin inter-neuronsof wt and HIPK2-KO mice. A-B-C Parvalbumin labeling in cerellar cortex of wt mice. Ob.20x. a-b-c Low magnification. Ob.2x. D-E-F Parvalbumin labeling in cerellar cortex of HIPK2-KO mouse. Ob.20x. d-e-f Low magnification. Ob.2x.

No differences in cell number have been observed for deep stellate cells (basket cells) and for outer stellate cells in HIPK2 KO mice (Figure 23.A).

Furthermore, has been observed no alteration in granule cells as identified NeuN antibody (Figure 23.A).

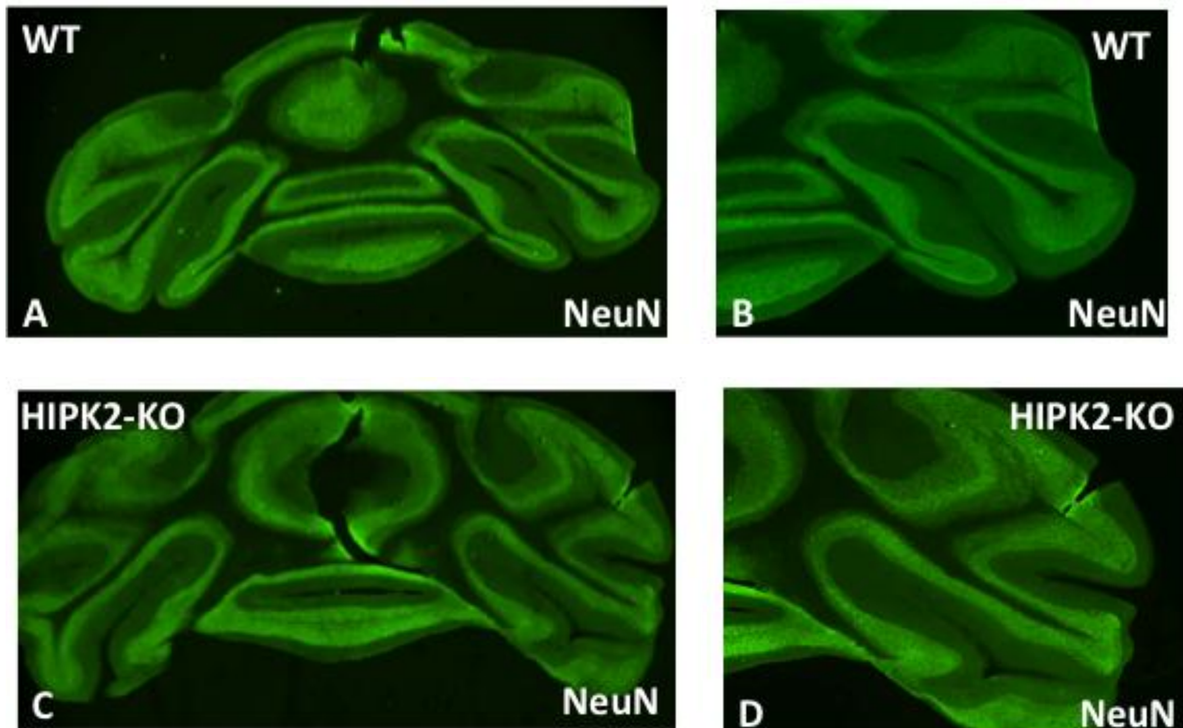



Figure 23.A-**Granule, deep stellate and outer stellate cells distribution in cerebellar cortex.** Confocal laser scanning microscopy (CLSM) images of single-label immunofluorescence for NeuN of wt and HIPK2-KO mice. A- NeuN labeling in cerebellar cortex of wt mouse. Ob.2x. B- NeuN labeling in wt mouse. Ob.4x. C-NeuN labeling in HIPK2-KO mouse. Ob.2x. D- NeuN labeling in HIPK2-KO mouse. Ob.4x.

### 4.4 Novelty induced exploration test reveals a hyperactive behavior in HIPK2 KO mice

Loco motor activity in an novelty induced exploration test was assessed at ages 5, 16 and 35 weeks of age. The statistical analysis included genotype, total distance traveled and age as main factors in a two way ANOVA. Groups of wild type or

HIPK2-KO mice were placed in experimental cage (Figure 24). The total distance traveled (m) over 30 minutes was recorded.

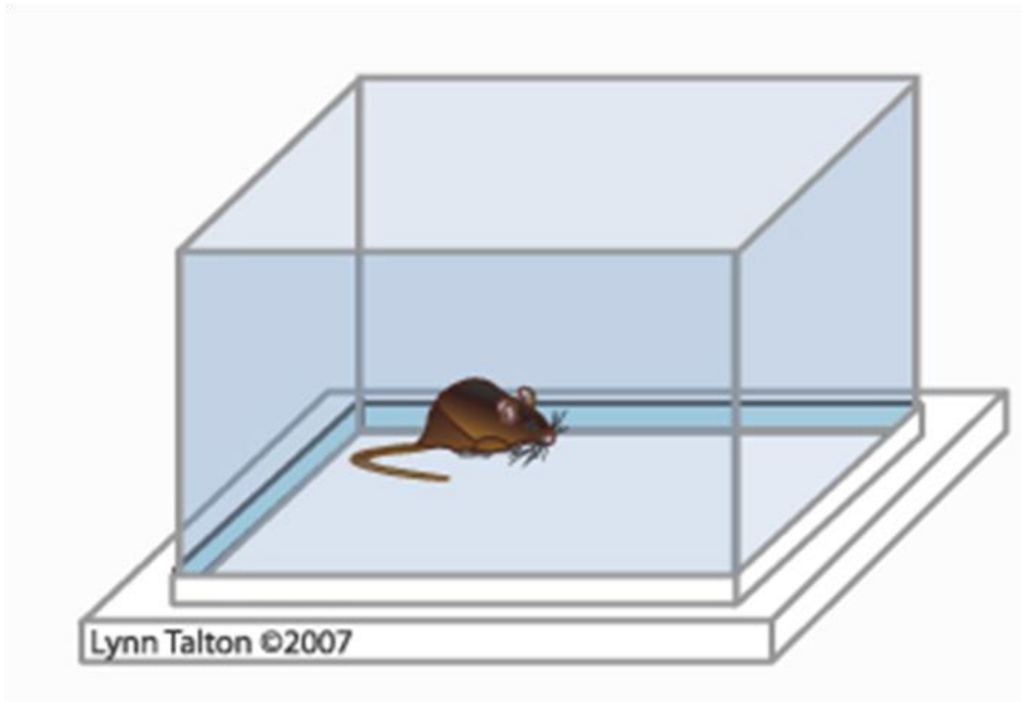
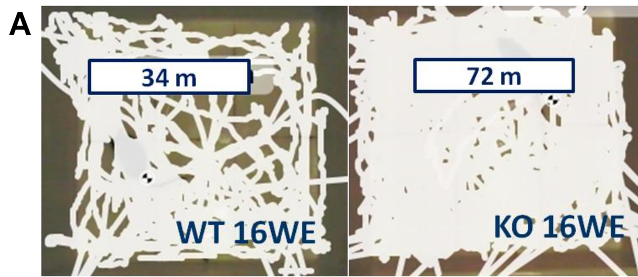



Figure 24- **Experimental cage of novelty induced exploration test**

The statistical analysis indicated an overall significant effect of genotype ($F(1,42)=33,58; P<0,0000$) and total distance traveled ($F(2,42)=21,73; P<0,0000$) instead there were no significant differences of age effect and a genotype X total distance traveled interaction. Post hoc analysis indicated that HIPK2-KO mice traveled a significantly greater distance than the WT mice in the first 10 minutes of the experiment at 16-35 weeks of age, while at 5 weeks of age the distance traveled increased but no significantly compared to WT mice at 5 weeks of age.

These data, expressed as a meters traveled in the first 10 minutes of experiment at weeks 5-16-35, are presented in Figure 25.



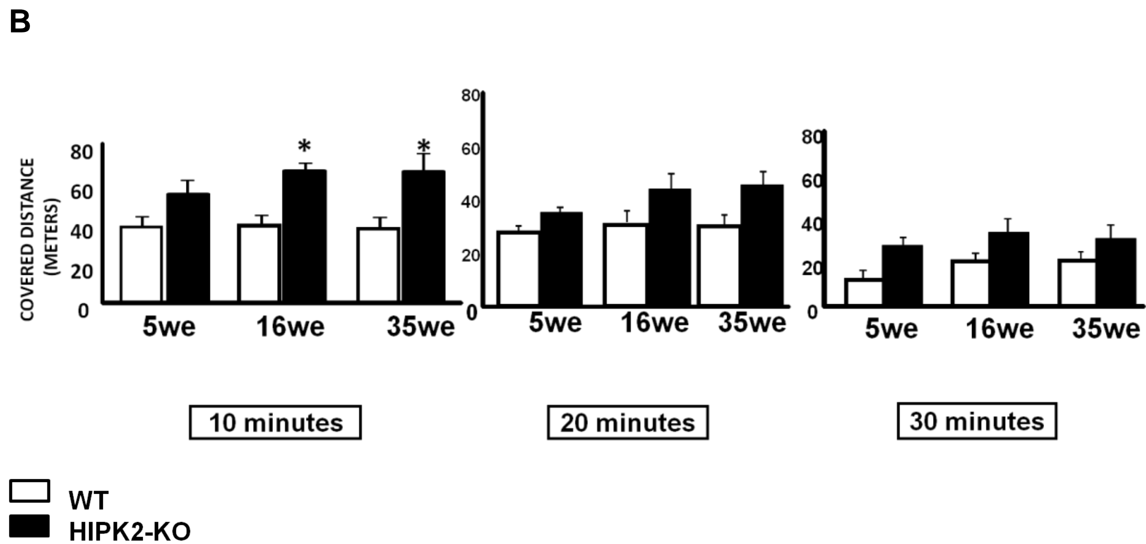




Figure 25-**HIPK2-KO mice walk more meters than wt at 16 and 35 weeks of age in novelty induced exploration test.** A- representative image of distance traveled of wt at 16 weeks of age and HIPK2-KO mice at 16 weeks of age
B- Average of distance (meters) traveled in the thirty minutes of the experiment. The significant effect was observed in the first ten minutes of experiment,

### 4.5 HIPK2-KO mice exhibit a muscle impairment in wire hanging test

In the wire hanging test were measured 1) the score, as previously described, 2) the time of latency and 3) time to walk the wire. (Figure 26).

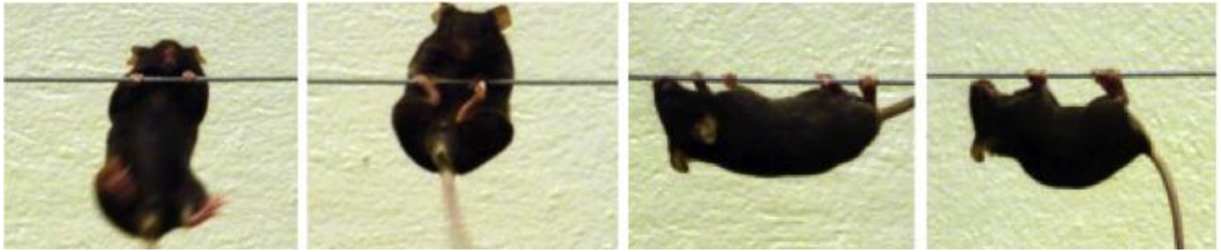
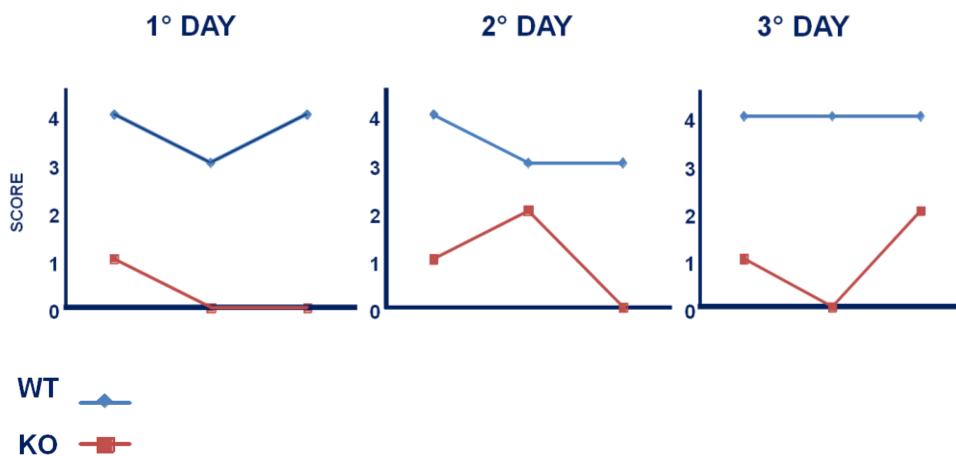


Figure 26- **Schematic representation of wire hanging test**. This image described the step of mice starting from hung onto the wire by two forepaws ending with hung onto the wire by all four paws plus tail wrapped.

In all three cases there were a strong difference between WT animals and HIPK2-KO mice. The time of latency (time to reach the right position) of all three day of training dramatically increased in HIPK2 KO mice (One way ANOVA; Genotype effect 1day $F_{1,16}=6,34$; 2day $F_{1,16}=7,22$; 3day $F_{1,16}=5,83$). The score are presented in Figure 27

**A**




**B**

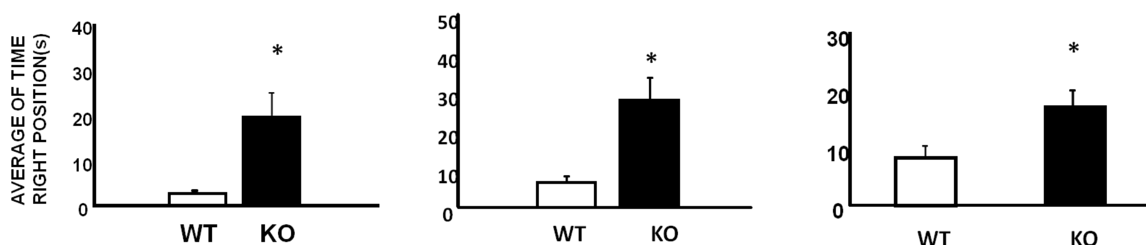


Figure 27-**In wire hanging test HIPK2 KO mice showed an impairment in the time spent to reach the right position.** A- Score of wire hanging test during three days of experiments in wt and HIPK2-KO mice.
B- Average of time to reach the right position during three days of experiments in wt and HIPK2-KO mice.

**4.6 HIPK2 KO mice show a walking awkwardly in Beam balance test**

The results of the beam balance also revealed that in HIPK2-KO mice increased significantly the traveling time on beam in the first 2 day of experiment (One way ANOVA Genotype effect: 1day $F_{1,16}=10,24$; 2day $F_{1,16}=4,88$) while the third day the traveling time increased but no significantly in HIPK2 KO mice compared with control group. The number of falls was showed in Figure 28

**A**

| GENOTYPE | NUMBER OF FALLS |
|---|---|
| WT | 2 |
| HIPK2-KO | 10 |

**B**

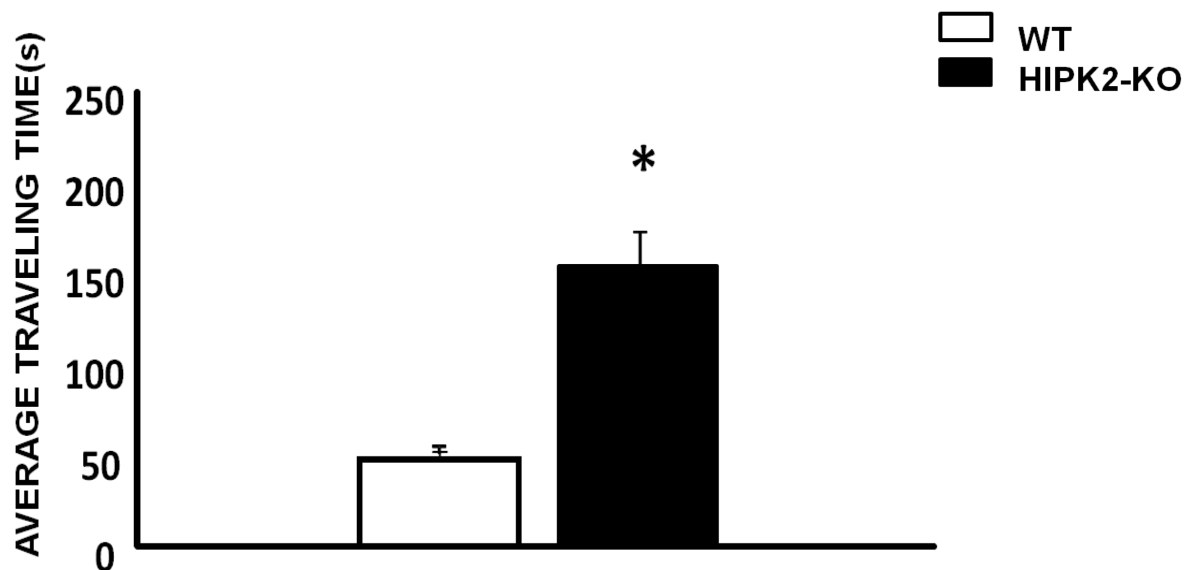



Figure 28- **HIPK2-KO mice showed a significant increase in the traveling time on beam and the number of falls in beam balance test.** A- Number of falls of wt and HIPK2-KO mice during three days of experiments
B- Average (three days) of traveling time on beam in wt mice and HIPK2-KO animals.

### 4.7 T-maze spontaneous alternation test revealed that absence of HIPK2 induces working and short term memory deficits.

Groups of wild type or HIPK2-KO mice were placed in a experimental cage (Figure 29) at ages 5, 16, and 35 weeks of age.

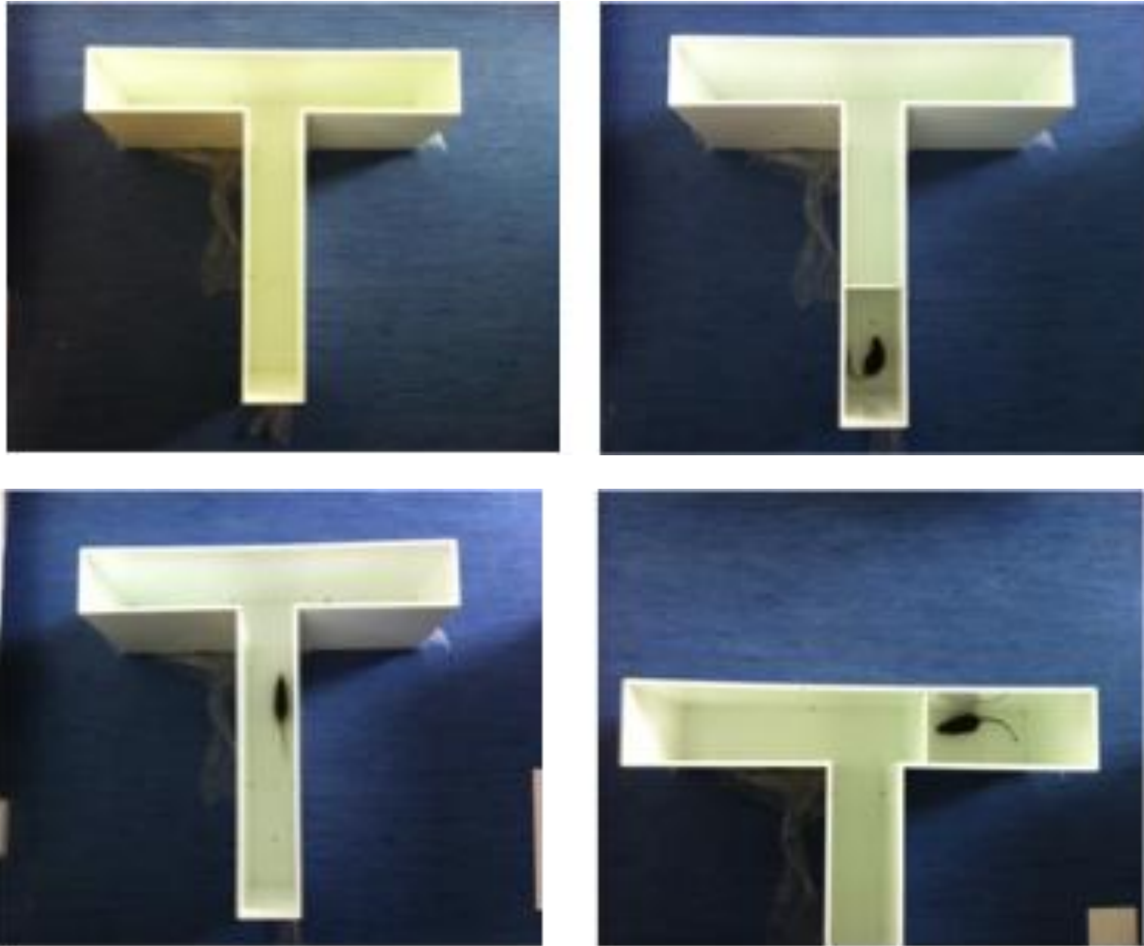


Figure 29- **Schematic representation of T-maze spontaneous alternation**.

Analysis of the percentage of alternation revealed a significant effect of genotype and age in HIPK2-KO mice (Genotype effect F1.18 = 37.71, age effect F2,18 = 10.74) (Figure 31). Also the analysis of time spent to decide revealed a significant effect of genotype (Genotype effect F1.18= 4.48) (Figure 30) instead we observed no significant differences in age effect and a genotype X time to decide interaction.

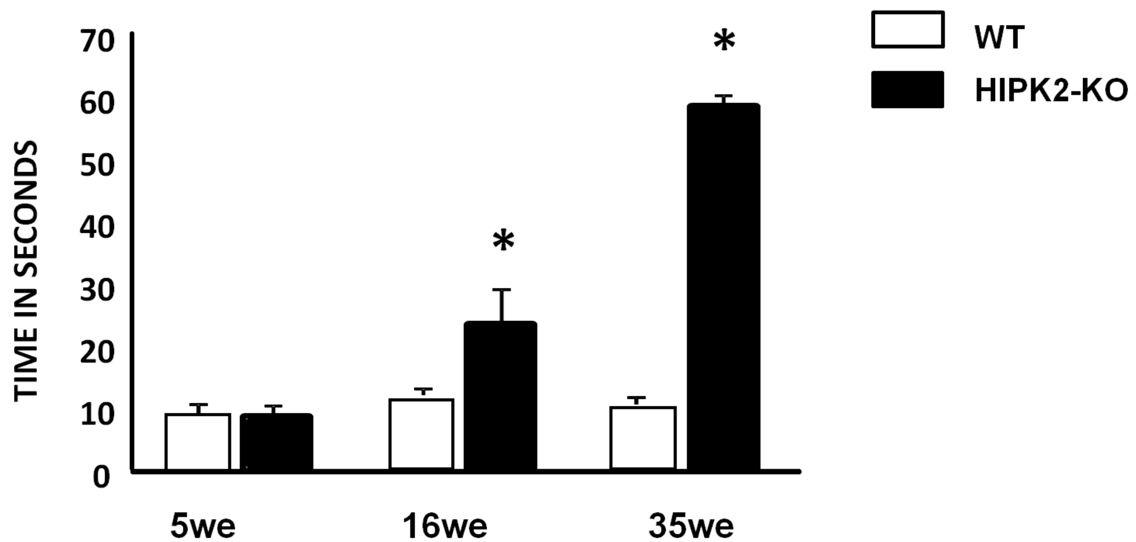



Figure 30- **In T-Maze spontaneous alternation the time spent to decide is longer in HIPK2 KO mice at 35 weeks of age compared to wt mice.**
Post hoc analysis showed that the time spent to decide was significant at 16-35 weeks of age compared to wt mice. In fact in HIPK2 KO mice at 35 weeks of age exceeds the maximum choice latency imposed at each trial.

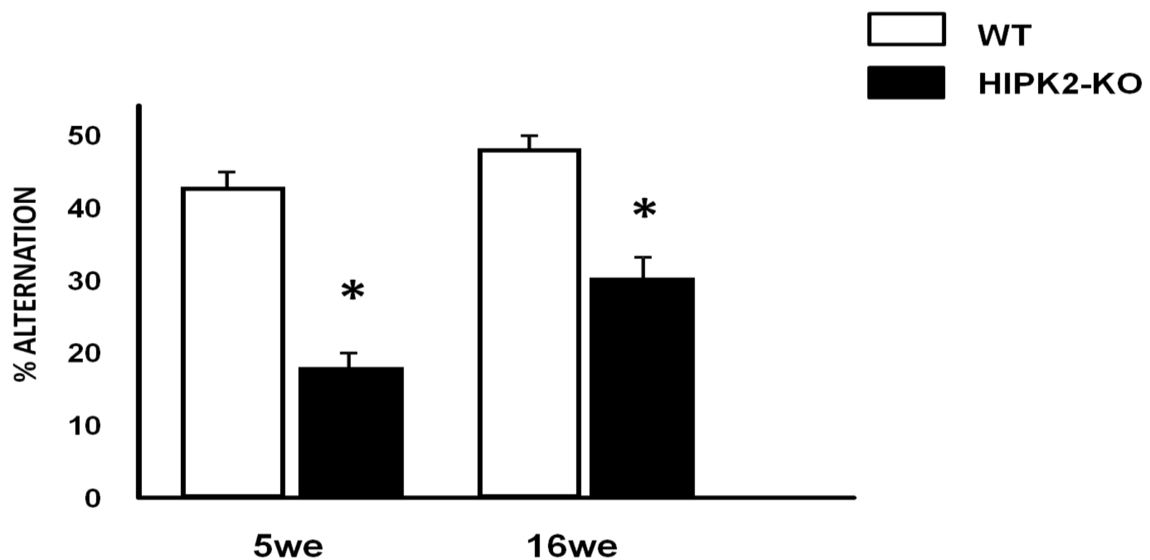



Figure 31-**T-maze spontaneous alternation test the number of changed direction is lower in 5-16 HIPK2 KO mice compared to wt mice**
Number of change direction (percentage of alternation) in wt and HIPK2-KO animals.

### 4.8 HIPK2 KO mice do not show alteration in long term memory and discriminative abilities analyzed in an object-recognition paradigm.

The object recognition test was assessed at ages 5, 16 and 35 weeks. (Figure 32)

Figure 32- **Schematic representation of object recognition test**

Overall, mutants displayed a significant reduction in the object exploration time, compared with control group (Figure 33).

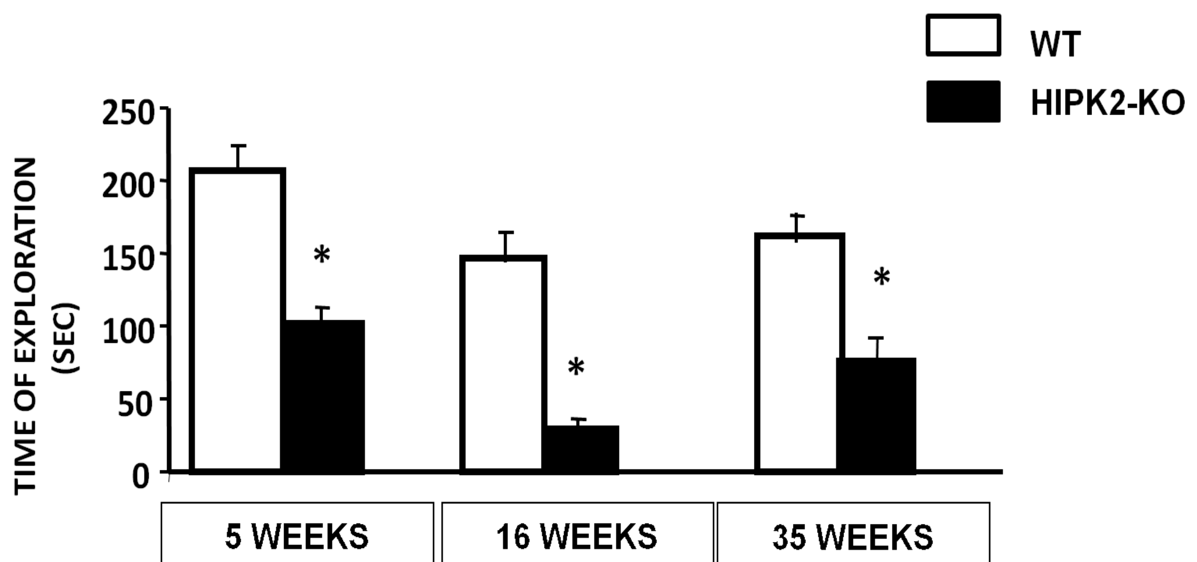



Figure 33-**In object recognition test the exploration time of two objects decreases in HIPK2 KO mice compared to wt mice.** Time of exploration in second during the training session in wt and HIPK2-KO mice at 5-16-35 weeks of age.

Moreover, on the retention test performed 24 hours after the first exposure to the familiar object, both WT and mutant mice showed a significant preference for the novel object. Post hoc analysis demonstrated that only significance differences were between WT mice at 5weeks of age and HIPK2 KO mice at 16weeks of age.

# 5 DISCUSSION

In the present study we describe the expression of HIPK2 protein and gene in the adult mice brains, and the role of HIPK2 in cognitive and motor behavior

The protein HIPK2 has many functions including the most important phosphorylation of the tumor suppressor p53, which activates a program of cell death.

This protein, indeed, has been thoroughly studied in cancers, in particular breast cancer and thyroid carcinoma, suggesting its important role in the process of carcinogenesis.

Recent studies have shown the expression of the gene HIPK2 during last stages of embryogenesis, especially at retina, muscle tissue and neuron levels.

Zhang et. al. have demonstrated that HIPK2 is essential for the survival of dopaminergic neurons. They have identified HIPK2 as a transcriptional coactivator that, interacting with the TGFbeta, regulates the preservation of dopaminergic neurons in the midbrain (21).

Our study revealed a strong expression of HIPK2 in cerebellum; these data were confirmed by real time PCR and immunofluroscence experiment.

Moreover we found some alterations in HIPK2-KO mice concerning short-term and working memory. In fact, in T-maze test, HIPK2 KO mice showed an extremely low number of changes of direction. While the control mouse had a percentage of alternation around 50%, the KO mouse showed a percentage around 20%. The significance of these results is that HIPK2 can be important in working memory.

A different scenario occurred while analyzing long term memory; in fact, in the object recognition test HIPK2 KO mice revealed no alteration in long term memory, the new object was indeed more explored than that familiar at all weeks of age.

Therefore the HIPK2 KO mice showed impairment in working memory while they did not have alteration in long term memory. These results can be justified with a strong decrease of the Gabaergic neurons of the cerebellar cortex in the Purkinge layer.

In particular, we observed a high reduction of parvalbuminergic and calbindin neurons of Purkinje layer.

Furthermore, these mice didn't have alteration in hippocampal neurons as revealed cell counting analysis, but the alteration was localized only in the cerebellum. These results demonstrate that the cerebellum have an important role in cognitive function and in particular in the so-called working memory.

Therefore, the traditional teaching that the cerebellum is purely a motor control device no longer appears valid. There is increasing recognition that the cerebellum contributes to cognitive processing and emotional control in addition to its role in motor coordination. Cerebral association areas that subserve higher order behavior are linked preferentially with the lateral hemispheres of the cerebellar posterior lobe in feedforward loops via the nuclei of the basis pontis, and in feedback loops from deep cerebellar nuclei via the thalamus. There are also reciprocal connections between the cerebellum and hypothalamus. These pathways facilitate cerebellar incorporation into the distributed neural circuits governing intellect, emotion and autonomic function (100).

The clinical relevance of these results is found in observations of cognitive and psychiatric manifestations of cerebellar lesions, and in the description of the cerebellar cognitive affective syndrome (CCAS) in patients with lesions confined to the cerebellum (101). The CCAS has subsequently been observed in adults and children with stroke, tumor, cerebellar degeneration, superficial siderosis, cerebellar hypoplasia and agenesis, and children born very preterm who have disproportionately small cerebella. They are characterized by impairments in

executive function (planning, set shifting, verbal fluency, abstract reasoning, working memory), spatial cognition (visual spatial organization and memory) and linguistic processing (agrammatism and dysprosodia) when the lesions involve the hemispheric regions of the cerebellar posterior lobes (102).

Following our observation we tested also balance and muscle strength. In fact, during the beam balance test HIPK2 KO mice could not coordinate forelimbs with hindlimbs and the mouse fell repeatedly and took much time to cross the beam.

The HIPK2 KO mice showed also impairment in the muscle strength in both forelimbs and hindlimbs. In fact, in most of the trials the mouse failed to come back to the original position.

This event could have been due, in part, to high dystonia that the animal showed in the hindlimbs (clasping test) when the animal was suspended by the tail (Figure 8) (85).

In conclusion, our work provides interesting focuses both on the connections between cerebellar structures and cerebral cortex (therefore cerebellum role in cognitive as well as motor behaviors) and on the role played by HIPK2 in the development and survival of cerebellum Gabaergic neurons.

In the next experiments we will deepen the role of HIPK2 in the development of the cerebellum and the expression of wnt/bcatenina pathway in embryos of HIPK2 KO mice. It has demonstrated, by recent studies, that the link between HIPK2 and bcatenin. In fact, in ex vivo mouse model, HIPK2 knockdown resulted in accumulation of beta-catenin, thereby potentiated beta-catenin-mediated cell proliferation and tumor formation. HIPK2 appeared to function as a novel negative regulator of beta-catenin (103).

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