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Targeted disruption of the *Pak5* and *Pak6* genes in mice leads to deficits in learning and locomotion

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ABSTRACT

PAK6 is a member of the group B family of PAK serine/threonine kinases, and is highly expressed in the brain. The group B PAKs, including PAK4, PAK5, and PAK6, were first identified as effector proteins for the Rho GTPase Cdc42. They have important roles in filopodia formation, the extension of neurons, and cell survival. *Pak4* knockout mice die in utero, and the embryos have several abnormalities, including a defect in the development of motor neurons. In contrast, *Pak5* knockout mice do not have any noticeable abnormalities. So far nothing is known about the biological function of *Pak6*. To address this, we have deleted the *Pak6* gene in mice. Since *Pak6* and *Pak5* are both expressed in the brain, we also generated *Pak5*/*Pak6* double knockout mice. These mice were viable and fertile, but had several locomotor and behavioral deficits. Our results indicate that Pak5 and Pak6 together are not required for viability, but are required for a normal level of locomotion and activity as well as for learning and memory. This is consistent with a role for the group B PAKs in the nervous system.

Introduction

The PAK kinases were originally identified as serine/threonine kinases that bind to the Rho GTPases Rac and Cdc42. Subsequently, the PAKs were shown to have both Rho GTPase dependent and Rho GTPase independent functions (Daniels and Bokoch, 1999; Knaus and Bokoch, 1998; Sells and Chernoff, 1997). There are 6 different PAKs in mammals and they fall into two categories, group A and group B, based on their amino acid sequences and structures. The group A PAKs include mammalian PAK1, PAK2, and PAK3 (Daniels and Bokoch, 1999; Knaus and Bokoch, 1998; Sells and Chernoff, 1997). Each of these protein kinases has an amino terminal regulatory domain and a carboxyl terminal kinase domain. Within the regulatory domain is a GTPase binding domain (GBD), which binds to activated Rac or Cdc42. The group B PAKs consist of PAKs 4, 5, and 6 (Jaffer and Chernoff, 2002). The group B PAKs also contain an amino terminal GBD and a carboxyl terminal kinase domain, but the rest of its sequence is completely different from the group A PAKs. Even the GBD and kinase domains of the group B PAKs have only approximately 50% identity with those of the group A PAKs. Of the group B PAKs, PAK4 is expressed ubiquitously in all tissues, while PAK5 and PAK6 are expressed only in some tissues, having especially high levels in the brain (Dan et al., 2002; Jaffer and Chernoff, 2002; Pandey et al., 2002; Yang et al., 2001). All members of group B family are highly homologous proteins, containing about 50% identity and 60% similarity. The only major differences between the three members of this family are in the regulatory domains, located between the GBD and kinase domains.

PAK4 was originally shown to be a link between Cdc42 and filopodia formation (Abo et al., 1998), and later PAK5 was also shown to promote the formation of filopodia (Dan et al., 2002). Filopodia formation is associated with the outgrowth of neurons, and it is interesting that expression of activated PAK5 leads to filopodia formation in neuroblastoma cells, as well as the formation of neurite like extensions (Dan et al., 2002). PAK5 can also bind and inhibit activity of MARK2 and thus regulate microtubule dynamics (Matenia et al., 2005; Timm et al., 2006). In addition to cytoskeletal organization, the group B PAKs also appear to have a number of other functions that may not be directly associated with cytoskeletal organization, including regulation of cell survival and proliferation, and induction of oncogene induced premature senescence

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(Cammarano et al., 2005; Cotteret and Chernoff, 2006; Cotteret et al., 2003; Gnesutta and Minden, 2003; Gnesutta et al., 2001). PAK6 was shown to have an important role in androgen receptor signaling (Lee et al., 2002; Yang et al., 2001), in a pathway that is not known to be linked to Rho GTPases at all (Lee et al., 2002; Yang et al., 2001). PAK6 can be stimulated by MKK6/p38 pathway and participate in the cellular response to stress-related signals (Kaur et al., 2005).

While a great deal is known about the role for the PAK kinases in cells, their biological and developmental roles are only recently being studied. Some important information about the PAKs in development has been obtained from studying their homologues in *Drosophila*, and such work suggests an important role for the PAKs in the developing nervous system. *Drosophila* PAK (DPAK) (Harden et al., 1996), a group A PAK, has been shown to be necessary for photoreceptor axon guidance (Hing et al., 1999), while Mushroom Body Tiny (MBT), a group B PAK, may regulate neuronal survival or proliferation (Melzig et al., 1998). Expression of PAK1 triggers neurite outgrowth in PC12 cells (Daniels et al., 1998), and activated PAK5, but not PAK1 promotes filopodia formation and neurite outgrowth in N1E-115 neuroblastoma cells (Dan et al., 2002). In humans, mutations in *Pak3* have been linked to nonsyndromic X-linked mental retardation (Allen et al., 1998; Bienvenu et al., 2000). Transgenic mice that express a dominant-negative allele which blocks all three group A *Paks* have an impairment in the retention of spatial memory, and this is associated with a decrease in the number of dendritic spines, and an increase in the size of synapse (Hayashi et al., 2004). The expression levels of group A PAKs were also shown to be reduced in Alzheimer's disease, and pharmacological inhibition of group A PAKs in adult mice leads to memory impairment (Zhao et al., 2006). These data suggest an important role for group A PAKs in the brain, including a role in the formation or retention of memories.

The generation of *Pak* knockout mice is an important way to study the biological phenotypes of the *Paks* in mammals. *Pak4* knockout mice are embryonic lethal prior to embryonic day 11 (Qu et al., 2003). *Pak4* null embryos most likely die due to cardiac (Qu et al., 2003) and/or extraembryonic tissue abnormalities (Tian and Minden, unpublished results), but interestingly they also have striking abnormalities in the nervous system (Qu et al., 2003). Most notably, spinal cord motor neurons and interneurons fail to differentiate and to migrate to their proper locations. Throughout the brain, the mice also display a lack of axonal outgrowth, and an abnormally thin neuroepithelium (Qu et al., 2003). In contrast to *Pak4*, which is ubiquitously expressed, *Pak5* expression is enriched in the brain. Interestingly, however, *Pak5* knockout mice are viable and fertile and do not have any noticeable phenotype (Li and Minden, 2003). Like *Pak5*, *Pak6* is also highly expressed in the brain. To investigate the biology of *Pak6* and to determine whether there is redundancy between the functions of *Pak5* and *Pak6*, in the present study we have developed *Pak6* knockout mice, and crossed these with *Pak5* null mice in order to generate *Pak5*/*Pak6* double knockout ($Pak5^{-/-};Pak6^{-/-}$) mice. $Pak5^{-/-};Pak6^{-/-}$ mice were viable and fertile, with no obvious physical abnormalities. However, when behavioral tests were carried out the knockout mice displayed a lower activity level than wild type mice, and a decreased level of aggression. They also displayed impaired cognitive function, including deficits in learning and memory retention. Our results provide evidence for critical involvement of PAK5 and PAK6 in locomotor activity and cognitive function.

Materials and methods

Construction of the targeting vector and generation of knockout mice

To genetically eliminate *Pak5* in mice, a construct was generated using the pPNT vector, which contains a neomycin expression cassette for positive selection, and an HSV-tk expression cassette for negative selection. Both 5′ and 3′ flanks for the construct were amplified by PCR using mouse embryonic stem cell DNA as a template. A 3.9 kb genomic fragment from 5′ untranslated region (UTR) of the mouse *Pak6* gene between two Xba1 sites was cloned into pPNT vector downstream of neo cassette as the 5′ flank. A 2.5 kb genomic sequence between the beginning of the exon II and an Xho1 site in the intron II was cloned upstream of neo as the 3′ flank. The neo cassette was in the opposite transcriptional orientation compared with the *Pak6* gene. After correct recombination, this vector causes 3.5 kb of genomic sequence including part of 5′UTR, exon I, intron I and 149 bp of exon II to be deleted and replaced by the neo expression cassette. The 3.9 kb 5′ flank for the targeting construct was PCR amplified using 5′ primer GCCTTTATCTAGATGATGAGAGATGACT and 3′ primer GCAAATACATCTAGAGTCACAAAAGAACTG, both corresponding to the 5′ UTR. The 2.5 kb 3′ flank was PCR amplified using 5′ primer ATATCTCGAGATGATCAGTGGGCTGCTGACCCCGAT, corresponding to the middle of exon II with the addition of an Xho1 site, and 3′ primer GGAAACTCGAGCTGCCACTTTAGGCTTGCTCAG within intron II. PCR was performed with Expand High Fidelity PCR system (Roche) according to the manufacturer's protocol.

The targeting construct was linearized with Not1 restriction enzyme and electroporated into the R1 embryonic stem (ES) cell line. This was carried out at Targeting Core facility at the University of Iowa. The R1 ES cell line is a hybrid derived from both the Parental and Steel substrains of 129/Sv. Two positive ES cell clones were expanded and verified for correct recombination with Southern blot analysis. Genomic DNA was isolated from ES clones and digested with Kpn1, EcoRV, Sph1, Hinc2 and Xmn1 restriction enzymes. Two external probes were amplified by PCR using mouse genomic DNA as a template and cloned into a TOPO-TA vector. Probes were sequenced for verification. A 1 kb genomic sequence between an Xmn1 and Xba1 sites was used as the 5′ probe. A 900 bp fragment between an Sph1 and EcoRV sites was used as 3′ probe.

$Pak6^{+/-}$ cells were injected into C57/BL6 blastocysts. Chimeric male mice displaying >50% coat color chimerism were bred to C57/BL6 females to generate F1 offspring. Germline transmission of the targeted *Pak6* allele was verified by PCR and Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Heterozygote progeny were interbred to obtain homozygote mice. Genotypes were verified by PCR and Southern blot analysis. Two lines of mice carrying the targeted *Pak6* allele in the germline were established from two independent ES cell clones.

Generation of $Pak5^{-/-};Pak6^{-/-}$ double knockout mice

$Pak6^{-/-}$ mice were crossed with $Pak5^{-/-}$ mice to obtain double heterozygous $Pak5^{+/-};Pak6^{+/-}$ progeny. These mice were crossed to obtain $Pak5^{+/-};Pak6^{-/-}$ mice, which were bred to obtain $Pak5^{-/-};Pak6^{-/-}$ mice. Genotypes were verified by PCR.

Genotyping by PCR

The targeted *Pak6* allele was detected by PCR with a forward primer; 5′GCTACCGGTGGATGTGGAATGTGT, corresponding to the neo sequence, and a reverse primer (5′-GAGGAAACCCCAGGTCATATACCT), corresponding to the sequence downstream of exon II. The *Pak6* wild type allele was detected with forward primer (5′-TCAGTTATCAGCTCCAACACCCTG), corresponding to the sequence in the beginning of exon II and the reverse primer described above. The targeted *Pak5* allele was detected by PCR with forward primer (5′-CTTCCTGACTAGGGGAGGAGTA), corresponding to the neo sequence and a reverse primer (5′-AGATGCATTGAGTGCTGGGGAA), corresponding to the sequence within the intron III. Wild type *Pak5* allele was detected by PCR with forward primer 5′-GCTTCCTCAGATCCATCCAAGGT and the reverse primer described above. The annealing temperature for both PCR reactions was 60 °C.

Antibodies and western blot analysis

Rabbit antibodies were raised against the amino acid sequence GHRQVPWPEPQSPQALP, located in the linker region of the mouse PAK6 protein (21 Century Biochemicals, MA). Antibodies were affinity purified. For Western blot analysis mouse brain and other tissues were removed, regions of the brain were dissected and then homogenized in 0.5–2 mL of modified RIPA buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EGTA, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 10% glycerol, 2 μg/mL aprotonin, 2 μg/mL leupeptin, 1 mM $Na_4P_2O_7$, 250 μM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 2 mM sodium orthovanadate). Concentration of SDS in the extraction buffer for PAK5 protein was 1%. Samples were centrifuged for 20 min at 13,000 ×*g* at 4 °C. Laemmli sample buffer was added to the aliquots containing same amounts of protein, and the samples were boiled at 95 °C for 5 min. Samples were electrophoresed on a 7% SDS-polyacrylamide gel and blotted electrophoretically to Immobilon membrane. PAK5 protein was transferred in the presence of 0.025% SDS in the transfer buffer. Membranes were blocked in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 5% nonfat milk. The following primary antibodies were used: antiPAK6 (1:100; 21 Century Biochemicals), antiPAK4 (1:1000; Cell Signaling). To detect PAK5 protein we used polyclonal rabbit antiPAK5 antibody, which we raised against full length, bacterially expressed mouse PAK5 protein. These antibodies were also affinity purified (Century 21 Biochemicals, MA). All blots were incubated with a secondary antibody conjugated to horseradish peroxidase (1:10,000) and developed using the enhanced chemiluminescence method (GE Healthcare). Protein concentration was determined using the BioRad protein assay.

Histological analysis

Double *Pak5*/*Pak6* knockout mouse and wild type mouse were anesthetized with 80 $mg \times kg^{-1}$ ketamine and 10 $mg \times kg^{-1}$ xylasine HCl and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. The dissected tissues were fixed in Boince overnight at room temperature. Samples were left in 70% ethanol overnight, dehydrated and embedded in paraplast (Fisher Scientific). 7–10 μm sections were cut on a Leitz 1512 rotary microtome. The tissues were stained with hematoxylin and eosin or cresyl violet and luxol blue.

Behavioral procedures

All mice used in the study were on mixed 129/Sv × C57BL/6 genetic background. 11 male double knockout mice and 10 wild type male mice were used throughout the battery of behavioral paradigms. All animals were born within two weeks of each other and testing began at approximately three months of age. An additional set of five 3 week old $Pak5^{-/-}$;$Pak6^{-/-}$ and five wild type mice was independently tested in the grip strength test. Initially, the animals were group housed in plastic shoebox cages with free access to food and water for approximately 60 days. They were then transferred to individual cages (20 cm × 10 cm × 12 cm), also with free access to food and water. The animals were housed in a temperature and humidity regulated room with a 12 h light/dark cycle. Body weights were recorded weekly. All of the experiments were conducted blind such that the experimenter could not distinguish the wild types from the knock-outs. All procedures were conducted in strict compliance with the policies on animal welfare of the National Institute of Health and the Animal Care and Use Committee at Rutgers University.

Open field test

On approximately day 80 locomotor activity was assessed for 30 min in a novel environment (42 × 22 × 14 cm Plexiglas box) with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 5 min. The chamber was wiped with alcohol to eliminate any scent left behind from the previous animal.

Rotorod performance

Mice were assessed for balance and motor coordination on a 6.0 cm diameter rotorod rotating at 12 revolutions per min with a maximum of 60 s per trial for a total of three trials. The latency until the animal fell from the rotating rod was recorded.

Hanging wire grip strength

At 3 weeks of age mice were placed on a grid wire surface. The grid was inverted and held 30 cm above a padded surface. Latency to fall was recorded with a maximum of 45 s for each trial.

T-maze—active avoidance

We tested mice approximately 120 days of age in a T-maze consisting of two 20 × 11 cm chambers connected to a 40 × 10 cm corridor with 18 cm high walls made of Plexiglas. The floor was made of stainless steel bars spaced 0.75 cm apart and connected to a shock generator except in the "safe" chamber. In each trial mice were placed in the start box with an initial interval of 20 s. A conditional stimulus tone accompanied by the opening of the start box door initiated the trial. The correct response, moving to the safe arm of the T-maze within 10 s, avoided the shock. Failure to make an avoidance response led to onset of a 0.8 mA foot shock, which could be terminated by moving to the safe arm as an escape response (also 10 s maximum). Each animal was given 8 sessions of 10 trials across 8 days. The type of response (avoidance or escape) and the latency for the animal to make either avoidance or escape response were recorded.

T-maze—passive avoidance

Mice at approximately 130 days of age were then switched to a passive avoidance condition where the previously safe side was now electrified and mice had to inhibit their entry into this arm. Each animal was given two 3 trial sessions across two consecutive days. Each trial consisted of a 20 second period when the tone was played. If the mouse entered the previously safe chamber, it received a shock and the trial was terminated. If the mouse avoided the previously safe chamber for the 20 second period, no shock was delivered. The latency to enter the previously safe chamber was recorded.

Elevated plus maze

At approximately 70 days of age mice were placed in an elevated plus maze consisting of two open arms and two closed arms 30 cm long and 9 cm wide that cross a neutral 5 cm × 5 cm central square. Closed arms had vertical shields raised from the edges. The entire apparatus was elevated 60 cm above the floor. Each animal was placed in the center of the maze and was given one 10-minute session to explore the maze. The number of entrances to closed arms, open arms, and jump-offs were recorded, as well as the number of fecal boli.

Social chamber

At approximately 160 days of age mice were placed in a social chamber (40 cm × 40 cm × 36.6 cm made of plexiglass and covered with brown cardboard). Two wired cylinders, 11 cm in diameter and 13 cm high, were located inside the chamber. Prior to the experimental sessions, the animal was placed in the middle of the chamber and was allowed to explore the chamber for 10 min. After the habituation period, a male C57BL/6 mouse was placed in the target

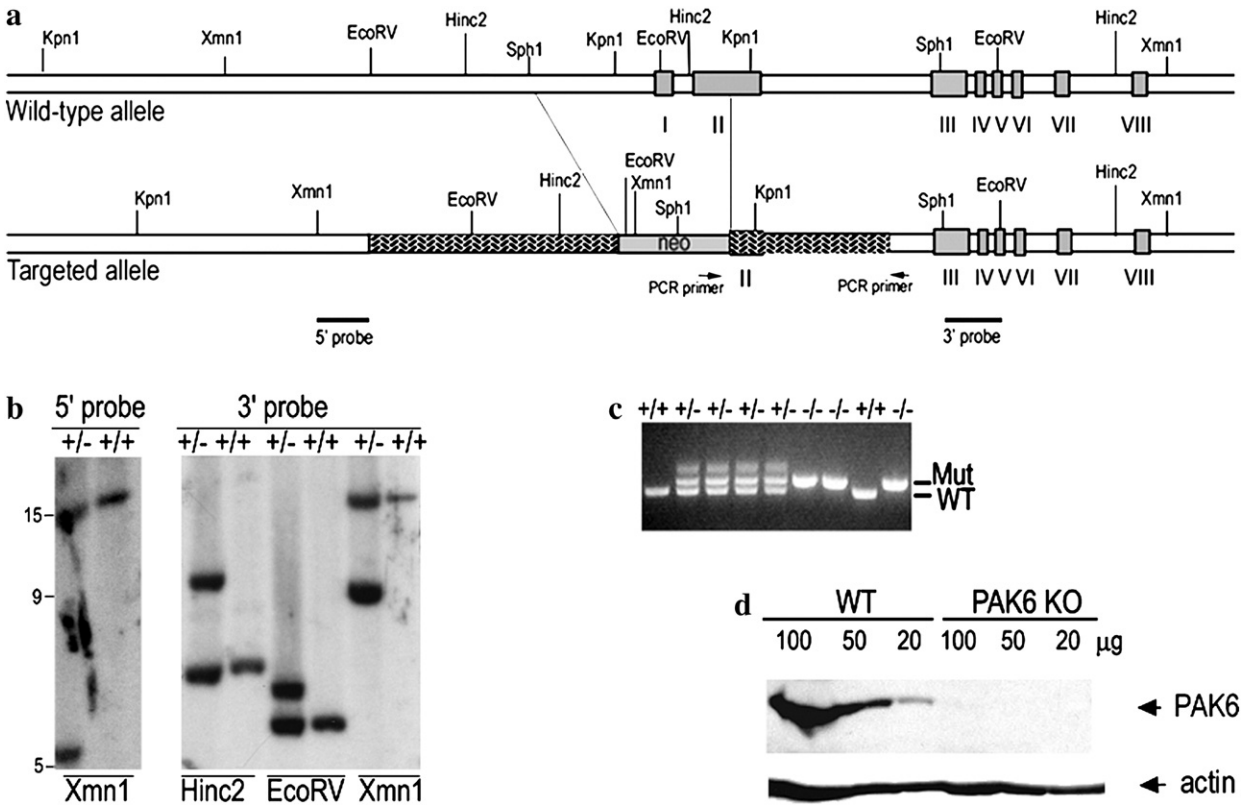

Fig. 1. Generation of Pak6 knockout mice. (a) Schematic representation of the wild type and targeted loci of the mouse *Pak6* gene. Exons I–VIII are depicted as shaded boxes. Exon I encodes the first 68 amino acids, including the GTPase binding domain (GBD). Exons III–VIII encode the kinase domain. The targeting construct was made to replace exon I and part of the exon II with the neomycin expression cassette. Homologous flanks in the construct used for targeting *Pak6* are shown as patterned areas. Primary screening for correct recombination was performed by PCR using primers surrounding the 3′ homologous flank of the construct. The locations of the PCR primers are indicated on panel a. Locations of the restriction sites used for secondary screening by southern blot are also indicated. (b) Southern blot analysis of genomic DNA from ES cell clones. Verification of correct recombination was done by Southern blot hybridization using external 5′ and 3′ probes illustrated in panel a. DNA isolated from ES cell clones transfected with the targeting vector, as well as from wild type ES cell clones was digested with the indicated restriction enzymes and analyzed by southern blot hybridization. The heterozygous (+/−) clone displays both wild type and recombinant DNA bands. (c) PCR analysis of mouse tail DNA isolated from +/+, +/− and −/− animals. Locations of PCR products for wild type and recombinant (mut) alleles are indicated. (d) Western blot analysis of PAK6 protein in lysates prepared from brains of wild type and $Pak6^{-/-}$ mice. The position of the 75 kDa band recognized by the anti PAK6 antibody is indicated. No expression of PAK6 could be detected in the brains of $Pak6^{-/-}$ mice. The same blot was also probed with anti actin antibody to verify equal loading.

cylinder, while the control cylinder was left empty. Each animal was allowed three 10-minute sessions across three consecutive days. The total number of contacts with both cylinders during the session was recorded.

Aggression test

At approximately 8 months of age the male mice were tested for aggressive behavior using the resident–intruder test. Resident mice were housed individually for two weeks before the procedure. The intruder mice were housed in cages at five mice per cage. The test consisted of placing a male intruder in the cage for one 30-minute session per day for each of three days. The latency periods prior to attack and the number of attacks were recorded.

Neurochemistry

Male mice at 30 and 120 days of age (10 mice per genotype) were sacrificed and cerebellum, hippocampus, striatum, and frontal cortex were dissected, snap frozen in liquid nitrogen, and stored until homogenization. All tissue was homogenized in 0.3 ml of 0.4 N perchloric acid with 0.1 mM EDTA. Homogenized samples were centrifuged at 20,000 ×g for 20 min at 4 °C and the supernatant was frozen in liquid nitrogen until it was analyzed. Supernatant was assayed for dopamine, serotonin and their metabolites using HPLC-electrochemical detection (Bioanalytical System, West Lafayette, Indiana). The mobile phase consisted of 0.1375 M sodium phosphate (dibasic), 0.0625 M citric acid, 5.0 mg EDTA, and 14% methanol with a flow rate of 0.7 ml/min (Halladay et al., 1998).

Culture of dissociated neurons

Primary cortical neurons were cultured as previously described in (Qian et al., 1998). Neurons were isolated from cerebral cortices of wild type, *Pak5* knockout, *Pak6* knockout and double *Pak5*/*Pak6* knockout mouse embryos at embryonic day 14.5 (E14.5). After dissection, cortices were enzymatically dissociated in 5 ml of prewarmed Neurobasal media (Gibco), containing 10 U/ml papain (Worthington), 1 mM glutamine, 1 mM sodium piruvate, 1 mM *N*-acetyl-cysteine (Sigma) and 15 µl of 4 mg/ml DNAse (Roche) at 37 °C for 30 min. Air was bubbled through the suspension for 30 s. The tube was placed on nutator for 30 min at room temperature. The tissues were then rinsed three times with Neurobasal media with gentle centrifugation. After the final wash, the tissue was dissociated by trituration using a plastic pipette two times, and then allowed to settle for 15 min before collecting the top fraction containing single cells. Cells were plated into 6-well plates coated with poly-L-lysine (50 µg/ml, Sigma) in Neurobasal medium containing B-27 and N-2 supplements (Invitrogen) with 10 U/ml penicillin, 10 µg/ml streptomycin and 0.5 µg/ml glutamine. The plating density was 3.5×10^3 cells/cm^2. Neurons were maintained in the same media, and half of the media was changed every 2 days. Plates were incubated in a CO_2 incubator at 37 °C. At least 20 neurons were used for quantification studies. In our experiments neuronal processes (neuritis) were defined as processes developed on

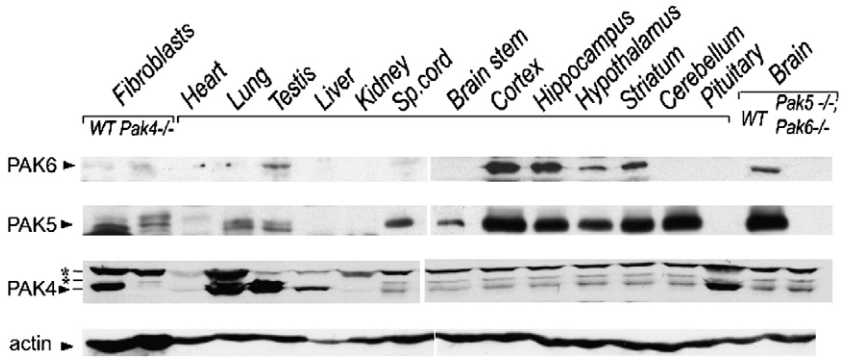

Fig. 2. Tissue specific expression pattern of group B PAKs in the mouse. Western blot analysis was performed on lysates from different tissues from a wild type mouse. Equal amounts of protein were separated by 7% SDS-polyacrylamide gel, transferred to Immobilon membrane and probed with anti PAK4, anti PAK5 or anti PAK6 antibodies. Both PAK5 and PAK6 are highly expressed in the mouse brain. The doublet seen in some lanes of the PAK5 blot may represent both phosphorylated and non-phosphorylated forms of PAK5. Blots were also probed with anti actin antibodies for loading control. Asterisks indicate nonspecific bands.

the cell body (soma) of neuron. A primary branch was defined as a branch developed on the neuronal process.

Immunocytochemistry

Cultured cortical neurons were fixed in 4% PFA with 4% sucrose in PBS 15 min at RT, permeabilized with ice cold 100% methanol at 4 °C for 20 min and blocked with 10% goat serum in calcium and magnesium containing PBS for 1 h at room temperature. Cells were stained overnight at 4 °C with a monoclonal antibody against β-tubulin III (1:100 dilution; Sigma) and Alexa555-conjugated Phalloidin (1:30 000 dilution; Molecular probes). Cells were washed four times with PBS and blocked with 10% goat serum in calcium and magnesium containing PBS for 30 min at room temperature. Cy™2-conjugated goat anti-mouse secondary antibodies (Jackson Laboratory) were used to detect β-tubulin III. After a 60-minute long incubation with secondary antibody, cells were washed three times in PBS and mounted with Crystal Mount mounting media (Biomeda). Cells were visualized with Zeiss fluorescent microscope.

Results

Generation of Pak6 knockout mice

To study the biology of *Pak6*, a targeting vector was engineered to remove exon I and part of exon II of the *Pak6* gene (Fig. 1a). In this vector the deleted regions were replaced with a neomycin resistance gene in the reverse orientation, which also contained stop codons in all three reading frames. The targeting construct was sequenced, linearized using a unique Not1 site, and electroporated into the R1 mouse embryonic stem (ES) cell line. A total of 215 clones were screened by PCR and two correct clones were identified. These clones were expanded and tested for correct recombination by Southern blot hybridization with 5′ and 3′ probes external to the cloned regions (Fig. 1b). After confirming that the recombination occurred in the correct locus, both clones were injected into C57/BL6 mouse blastocysts and implanted into pseudopregnant females. Germline transmission was achieved for several male and female chimaeras. Heterozygous mice were intercrossed to produce animals with a homozygous mutation of *Pak6* (Fig. 1c). As expected no PAK6 protein was detected in the brains of homozygous *Pak6* null mice (Fig. 1d). *Pak6* knockout mice were viable, fertile and did not have any visible abnormalities.

Tissue specific expression patterns of PAK5 and PAK6

Because the *Pak6* knockout mice did not have an observable phenotype, we were interested in determining whether there is redundancy between *Pak5* and *Pak6*. Like *Pak6*, *Pak5* is also a group B *Pak*, and highly expressed in the brain. Likewise, *Pak5* knockout mice also have no visible phenotype. Northern blot analysis of the human tissues showed that *Pak6* mRNA is highly expressed in testis and various regions of the brain (Lee et al., 2002; Yang et al., 2001). However, there is currently no information available regarding expression of *Pak6* mRNA in mouse tissues, and there is no information about the expression pattern of PAK6 protein in either mice or humans. We therefore carried out western blot analysis of different mouse tissues to determine which tissues contain PAK6 protein. To our surprise, a relatively low amount of PAK6 protein was

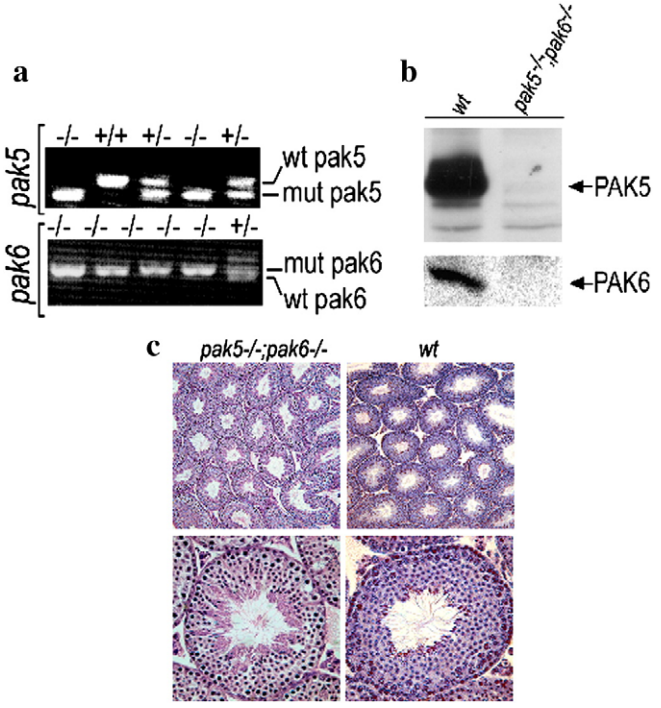

Fig. 3. Generation and analysis of $Pak5^{-/-};Pak6^{-/-}$ mice. (a) PCR analysis of genomic DNA isolated from tails of pups generated from the $Pak5^{+/-};Pak6^{-/-} \times Pak5^{+/-};Pak6^{-/-}$ intercross. Two separate PCR reactions were performed using primers to amplify part of the *Pak5* or *Pak6* genomic loci, as described in the Materials and methods section. Locations of the bands corresponding to the PCR products for wild type or recombinant *Pak5* or *Pak6* loci are indicated. (b) Western blot analysis of PAK5 and PAK6 proteins in lysates prepared from the brains of wild type and $Pak5^{-/-};Pak6^{-/-}$ mice. No PAK5 or PAK6 protein was detected in the lysates prepared from the cortexes of $Pak5^{-/-};Pak6^{-/-}$ mice. (c) Histological analysis of testes of wild type and $Pak5^{-/-};Pak6^{-/-}$ mice. 10 μm sections of the testes were stained with hematoxylin and eosin and analyzed under microscope to visualize morphology. No abnormalities were found in the testes of $Pak5^{-/-};Pak6^{-/-}$ mice.

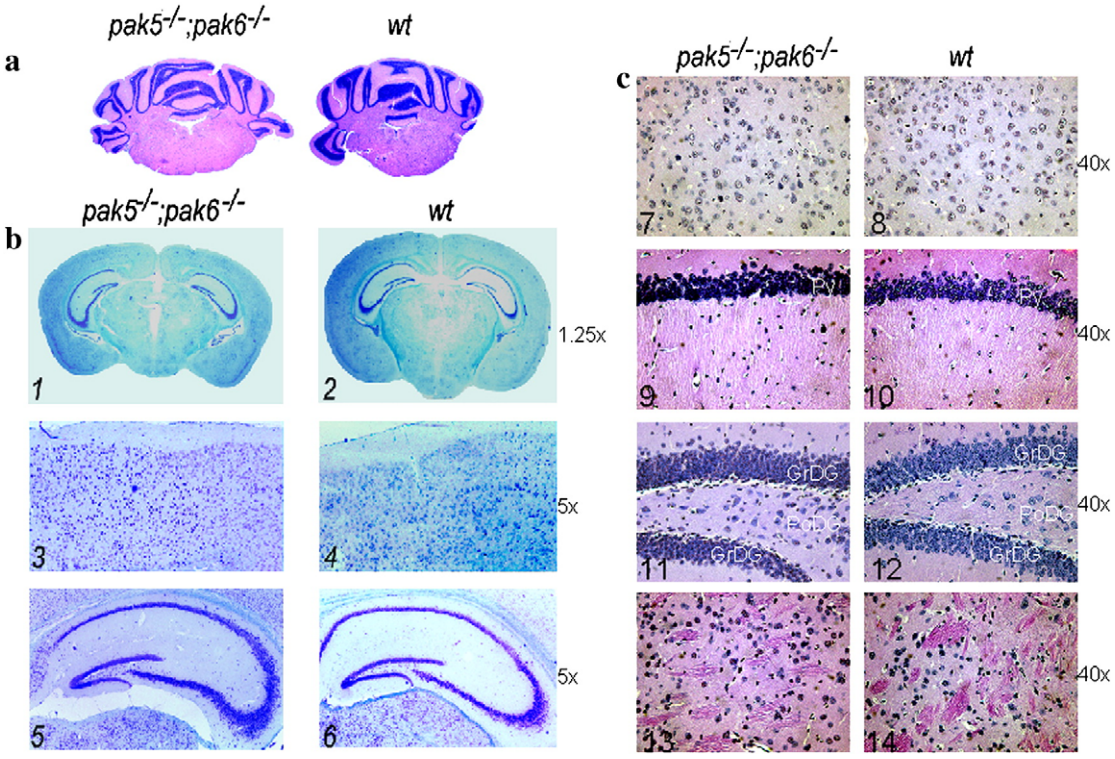

Fig. 4. Histological analysis of the brains of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. 7 μm coronal sections of the brains of wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice were stained and analyzed under the microscope, in order to visualize morphology. (a) Sections from the cerebellum stained with hematoxylin and eosin. (b) Sections from the diencephalon stained with luxol blue and cresyl violet. Images at higher magnification (5×) of the cerebral cortex (panels 3,4) and hippocampus (panels 4,5) are shown. (c) 40× magnification images of cortex (panels 7,8), hippocampus (panels 9, 12) and striatum (panels 13,14) stained with haematoxylin and eosin. (Py) — pyramidal cell layer of hippocampus, (GrDG) — dentate gyrus granule cell layer and (PoDG) — polymorphic layer of dentate gyrus. No gross abnormalities were detected in the brains of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice.

detected in the testis. The majority of the PAK6 protein was found to be in neural tissues (Fig. 2). Mouse brain was the organ containing the most PAK6, while spinal cord also had low amounts of the protein. Cortex and hippocampus were the areas of the brain with the highest levels of PAK6, while striatum and hypothalamus had intermediate amounts of PAK6. No PAK6 was detected in the mouse cerebellum or pituitary gland (Fig. 2).

Next we assessed the expression of PAK5 protein in various tissues. We found that PAK5 was abundant in the brain, and also expressed at very low levels in the heart, lung, testis and in the spinal cord. Within the brain PAK5 was found in the cortex, hippocampus, striatum, hypothalamus and cerebellum (Fig. 2). Thus, both PAK5 and PAK6 are found highly expressed in several areas in the brain: cortex, hippocampus and striatum. It is therefore possible that redundancy between these two proteins prevent phenotypic abnormalities in the knockout mice. We also examined the levels of mouse PAK4 protein. PAK4 was expressed at low levels in almost every tissue tested, as expected, although we detected significantly higher expression in the lungs, testis, and pituitary gland (Fig. 2).

Generation of double Pak5/Pak6 knockout mice

Because of the evolutionary homology between PAK5 and PAK6 and their overlapping expression patterns, the absence of an overt phenotype in the null *Pak5* or *Pak6* mice could be due to functional redundancy between the proteins. To circumvent this, we sought to genetically eliminate both proteins in the mouse and generate double *Pak5*/*Pak6* null (*Pak5*$^{-/-}$;*Pak6*$^{-/-}$) mice. These were obtained by crossing *Pak5* knockout mice with *Pak6* knockout mice (as described in Materials and methods). Both *Pak5* (chromosome 2, F3/G1) and *Pak6* (chromosome 2, E5) genes are located on the chromosome 2 in the mouse and separated by 17.6 Mb of genomic sequence. The efficiency of genomic recombination for such proximal genes was extremely low, but after continued breeding we obtained *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Genotyping of the progeny from the last breeding intercross is shown in Fig. 3a. Western blot analysis of the brains and cortexes confirmed that resultant mice are indeed double knockouts since both PAK5 and PAK6 proteins are absent in these tissues (see Fig. 3b). *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice were also viable and fertile. PAK6 is known to be a binding partner for androgen receptor, which downregulates its activity upon binding. Therefore, despite normal fertility in double knockout mice, we performed histological analysis of the male reproductive organs. No morphological abnormalities were detected in the testes from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. All tissue components in the testes were intact, and abundant primary spermatocytes and mature spermatozoa were observed (Fig. 3c).

Histological analysis of the brains did not reveal any gross abnormalities in the structures of any regions within the brain (Fig. 4). Coronal sections through the cerebellum were stained with hematoxylin and eosin. Proper development was evident, as well as the characteristic organization of foliae (Fig. 4a). The cortex and hippocampus, which normally contain high levels of PAK5 and PAK6, were analyzed by staining with cresyl violet to visualize neurons, and luxol blue to assess myelin fibers (Fig. 4b). No significant differences were found in the architecture of the cortex or the hippocampus or in the myelination of these regions. In the cortex, all of the layers were developed and in their correct positions. Myelination of these regions of the brain also appeared normal. Since both PAK5 and PAK6 proteins have been found in adult mouse cortex, hippocampus, and striatum, these structures were analyzed by staining coronal sections with hematoxylin and eosin and visualizing them under higher magnification (Fig. 4c). However, as shown for other areas of the central nervous system, neither cyto-architectural nor cytological abnormalities were evident. Higher magnification revealed a well developed cortex with no indication of cell loss, and normal distribution of neurons (Fig. 4c 7,8). The pyramidal cell layer of the hippocampus (Py) was developed

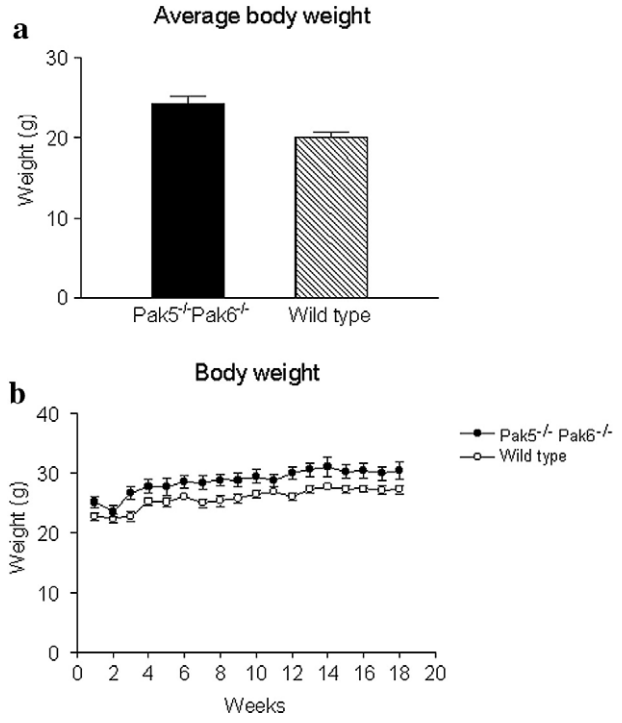

Fig. 5. Body weight of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. (a) Average weights of adult wild type ($n=10$) and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ ($n=11$) mice. $p=0.026$. (b) Changes in body weight in the two groups of mice over time. Animals were monitored over 4.5 months. Values are mean ± SEM.

normally (Fig. 4c 9,10). The dentate gyrus has normally developed granule cell layer (GrDG) and was densely populated with typical large neurons in the polymorphic layer (PoDG) (Fig. 4c 11,12). The striatum was organized in a normal fashion, showing the presence of both patch and matrix compartments and a normal distribution of neurons (Fig. 4 13,14). Thorough macroscopic analyses revealed a normally developed brain in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice.

Increased body weight in Pak5$^{-/-}$;*Pak6*$^{-/-}$ *mice*

The only noticeable physical difference between the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice and wild type mice was a slight difference in body weight. At 2.5 months old, the average body weight of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mouse was slightly higher then that of wild type mouse (Fig. 5a). The weights of mice were monitored throughout 4.5 months, and the results revealed that the average body weight of the double knockout mice continued to be somewhat higher than that of their wild type counterparts (Fig. 5b).

Deficits in locomotor activity and strength in the Pak5$^{-/-}$;*Pak6*$^{-/-}$ *mice*

Since Pak4, the founding member of the group B Pak family has been associated with motor neuron development (Qu et al., 2003), we carried out tests to study motor activity in the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Three tests were used to study locomotor activity and strength. These were the open field test, the rotorod test, and the hanging wire grip strength test. In the open field test the mice were tested for baseline locomotion and exploratory behavior. Since the environment was novel, wild type mice initially showed very high levels of locomotor activity, which decreased with time within the test session. *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice, when compared with wild type mice across 5-minute intervals, were much less active in each interval and did not demonstrate high activity even at the beginning of the exploration (Fig. 6a). The results showed a statistically significant decrease in locomotion of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice compared to wild type control mice. The total cumulative activity count of the double knockout mice was also significantly lower when compared with wild type mice (Fig.

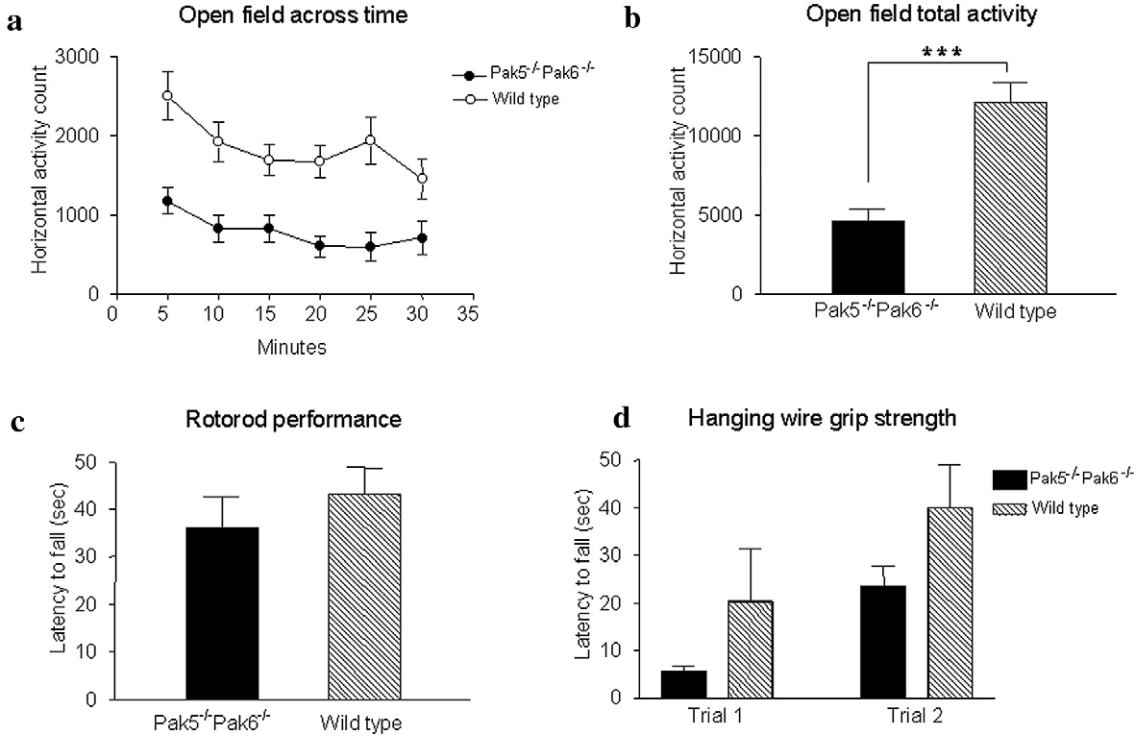

Fig. 6. Impaired locomotor activity in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. (a) Decrease in horizontal motor activity in adult *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Animals were placed in the activity box and activity level was recorded for every 5 min during a 30-minute period. (Wild type, $n=10$ mice; *Pak5*$^{-/-}$;*Pak6*$^{-/-}$, $n=11$ mice). (b) Decreased average level of motor activity in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice in the open field test. ***$p<0.001$, Student's t test. (c) Rotorod performance of wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. The average amount of time the mice spent on the rotating rod before falling is depicted. (d) Decreased grip strength in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Grip strength ability of 3 week old wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice was measured using the hanging wire test ($n=5$ mice for each genotype). Values are mean ± SEM. $p=0.027$ by two-way ANOVA test, genotype effect.

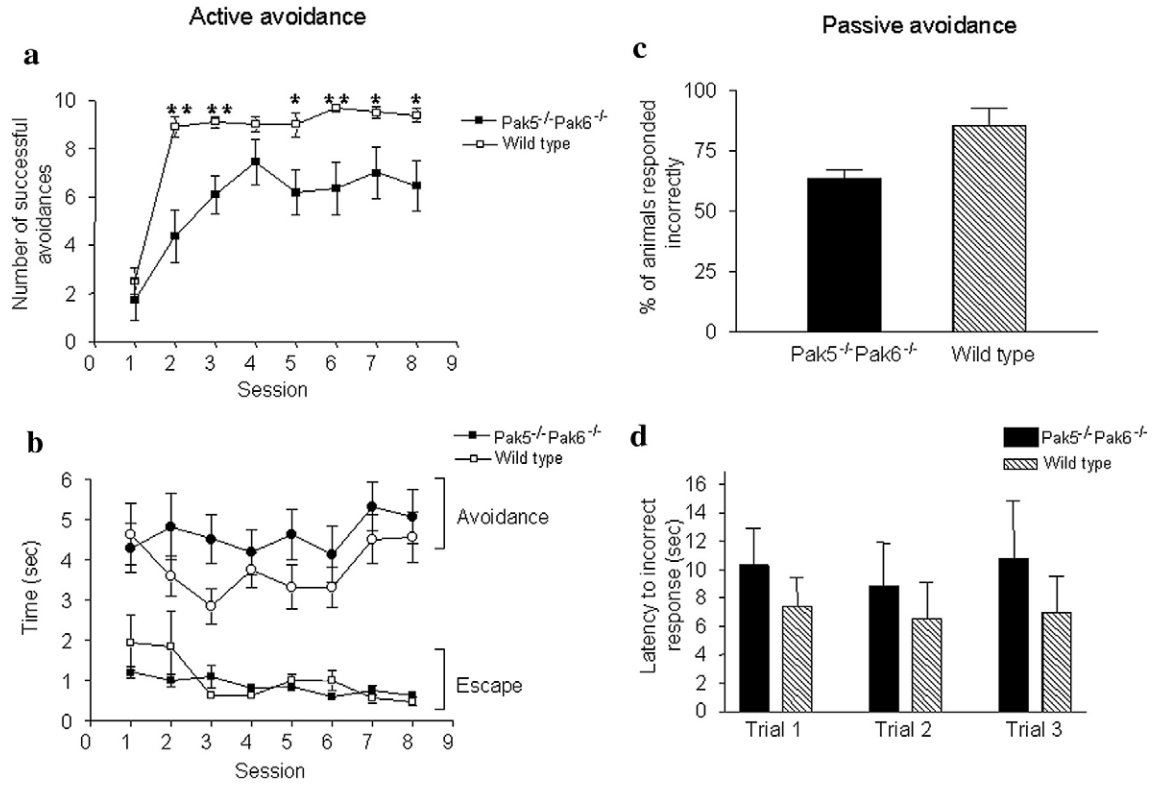

Fig. 7. Impaired learning and poor memory retention in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. (a, b) Active avoidance test. (a) Decreased number of successful avoidances in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ ($n=11$) versus wild type ($n=10$) mice in the T-maze test. Active avoidance behavioral responses are indicated as the number of mice within each genotype, which successfully avoided foot shock. $^{**}p<0.01$, $^{*}p<0.05$, Student's t test. (b) Increased foot shock avoidance time and normal escape time in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice as compared to wild type mice. (c, d) Passive avoidance test. (c) Passive avoidance test in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice ($n=11$) compared to wild type mice ($n=10$). Behavioral responses are indicated as the number of mice that responded incorrectly within each group. (d) Longer latency to incorrect response in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Values are mean ± SEM.

6b). Single knockout mice (*Pak5* or *Pak6*) behaved similarly to the wild type mice in this test (see Supplemental Fig. 1a). The results of the open field test strongly suggest a deficit in a motor function in the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice.

The rotorod test was used to evaluate balance and motor coordination in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. The time it took to fall from the rod was similar for both the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice and wild type mice in three trials. The average latency for falling was 42 ± 2.5 s for wild type mice and 38 ± 3 s for *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice (Fig. 6c). The results suggest that balance and coordination were not affected by deletion of *Pak5* and *Pak6*.

Grip strength was measured as the amount of time a mouse is able to remain suspended from a grid wire surface. Mice of both genotypes showed an improvement in hanging wire grip strength in their second trial compared with their first. However wild type mice performed significantly better in both trials and it took them longer to fall from a suspended wire compared to *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice (Fig. 6d). A two-way ANOVA test revealed significant effects of genotype on grip strength ($p=0.027$), where the wild type mice had greater grip strength. Single knockout mice (*Pak5*$^{-/-}$ or *Pak6*$^{-/-}$) behaved similarly to the wild type mice (See Supplemental Fig. 1b).

Learning and memory are impaired in Pak5$^{-/-}$;*Pak6*$^{-/-}$ *mice*

Since *Pak5* and *Pak6* are expressed in the brain, we decided to determine whether the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice had any cognitive, or emotional deficits. To assess possible cognitive dysfunctions we used the T-maze test. Performance in the active avoidance component of the T-maze improved over the different sessions, but the double knockouts acquired the correct avoidance response at a much slower rate than the wild type mice and performed significantly more poorly in most of the sessions (Fig. 7a). Wild type mice learned to avoid the foot shock and reached the peak of performance by the second session, while *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice learned to avoid the foot shock only by the fourth session. Furthermore, the maximum correct performance of the group of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice appeared to plateau at a significantly lower level (on average 70% of mice learned to avoid the shock compared with 90% of the wild type mice). By session 5 and beyond, the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice had a consistently lower number of successful avoidances compared with the control mice. When the experiments were carried out with single knockout mice we found that *Pak5* knockout mice also had a lower level of successful avoidances by session 5, but not as dramatic as the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$

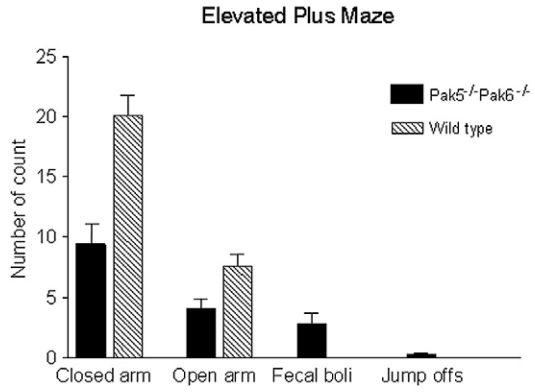

Fig. 8. Anxiety in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Performance in the elevated plus maze test was used as a measure for anxiety behavior in wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. The graph shows the number of entrances into the closed arms, and the number of entrances into the open arm in a 10-minute period, the number of fecal boli produced in a 10-minute period, and the number of jump-offs in a 10-minute period. *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice entered the closed and opened arms less times than wild type mice. The number of times *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice defecated was slightly higher than in wild type mice. The number of jump-offs from the maze was insignificant. Values are mean ± SEM.

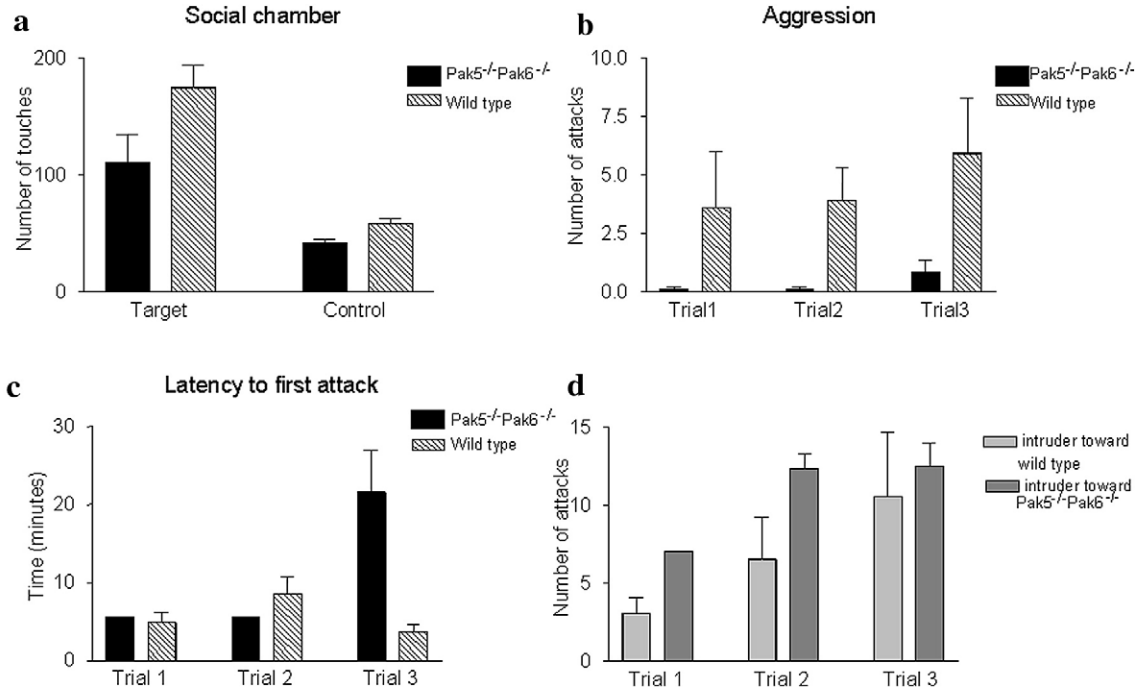

Fig. 9. Normal social behavior and decreased aggression in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. (a) Normal social behavior of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Behavior of wild type (n=10) and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ (n=11) mice in a social chamber was monitored for 10 min. The number of touches of the cylinder containing the novel mouse or an empty cylinder was measured and plotted. (b, c) Decrease in aggressive behavior in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice in the resident–intruder test. (b) Number of resident's bite attacks toward the intruder mouse within the 30-minute session is shown. (Wild type, n=10 mice; *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ n=11 mice). (c) The latency to the first attack by the resident mouse in the same test is depicted. (d) The number of intruder's attacks toward the resident wild type or *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mouse in the same test is shown. Data shown as mean±SEM.

mice. *Pak6* knockout mice behaved similarly to wild type mice (See Supplemental Fig. 2). As seen in Fig. 7b, double knockout mice also had a longer avoidance time than wild type mice.

In the passive avoidance component, the shock circuitry of the T-maze is reversed, and the original correct response of moving to the safe chamber becomes the incorrect response. During the previous

Table 1
Levels of neurotransmitters in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ (KO) and wild type (WT) mice

Genotype	Age (days)	DA	DOPAC	HVA	5HT	5HIAA
Frontal cortex						
WT	30	1.9 (0.2)	0.2 (0.02)	0.3 (0.02)	0.5 (0.01)	0.2 (0.007)
	120	4.5 (0.4)	0.5 (0.03)	0.7 (0.05)	0.8 (0.05)	0.2 (0.02)
KO	30	2.2 (0.1)	0.3 (0.01)	0.4 (0.02)	0.5 (0.01)	0.2 (0.01)
	120	3.9 (0.4)	0.3 (0.03)	0.6 (0.08)	0.8 (0.05)	0.2 (0.03)
Hippocampus						
WT	30	0.008 (0.001)	0.006 (0.004)	0.004 (0.0004)	0.05 (0.004)	0.03 (0.002)
	120	0.01 (0.003)	0.003 (0.0004)	0.005 (0.0008)	0.08 (0.004)	0.05 (0.004)
KO	30	0.01 (0.003)	0.002 (0.0002)	0.004 (0.0004)	0.05 (0.002)	0.04 (0.003)
	120	0.01 (0.003)	0.004 (0.0008)	0.006 (0.0005)	0.08 (0.005)	0.05 (0.003)
Striatum						
WT	30	10.1 (0.3)	0.9 (0.04)	1.4 (0.04)	0.4 (0.01)	0.4 (0.01)
	120	15.7 (0.9)	1.02 (0.06)	2.0 (0.1)	0.7 (0.04)	0.5 (0.02)
KO	30	8.7 (0.3)	0.8 (0.04)	1.4 (0.04)	0.5 (0.02)	0.5 (0.02)
	120	16.2 (0.8)	0.9 (0.2)	2.1 (0.2)	0.8 (0.02)	0.6 (0.05)
Hypothalamus						
WT	30	0.4 (0.03)	0.1 (0.007)	0.1 (0.007)	1.2 (0.03)	0.4 (0.01)
	120	0.7 (0.08)	0.2 (0.01)	0.2 (0.02)	1.6 (0.1)	0.5 (0.02)
KO	30	0.5 (0.1)	0.1 (0.01)	0.6 (0.3)	1.1 (0.03)	0.5 (0.02)
	120	0.8 (0.07)	0.2 (0.02)	0.3 (0.04)	1.8 (0.04)	0.6 (0.05)
Brain stem						
WT	30	0.1 (0.004)	0.05 (0.007)	0.07 (0.01)	1.0 (0.05)	0.4 (0.03)
	120	0.1 (0.007)	0.08 (0.006)	0.1 (0.004)	1.1 (0.05)	0.5 (0.02)
KO	30	0.1 (0.01)	0.1 (0.009)	0.1 (0.01)	1.02 (0.1)	0.6 (0.06)
	120	0.1 (0.01)	0.05 (0.003)	0.06 (0.007)	0.9 (0.05)	0.5 (0.04)

The concentration of neurotransmitters in μg/g wet tissue weight detected in the indicated tissues from brains of wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Values are expressed as mean (SEM). Neurotransmitters tested were dopamine (DA), 3,4-dihydroxyphenylacetic acid, DOPAC (a dopamine metabolite), homovanillic acid (HVA) (a dopamine metabolite), serotonin (5HT), and 5-hydroxyindoleacetic acid (5HIAA) (a serotonin metabolite).

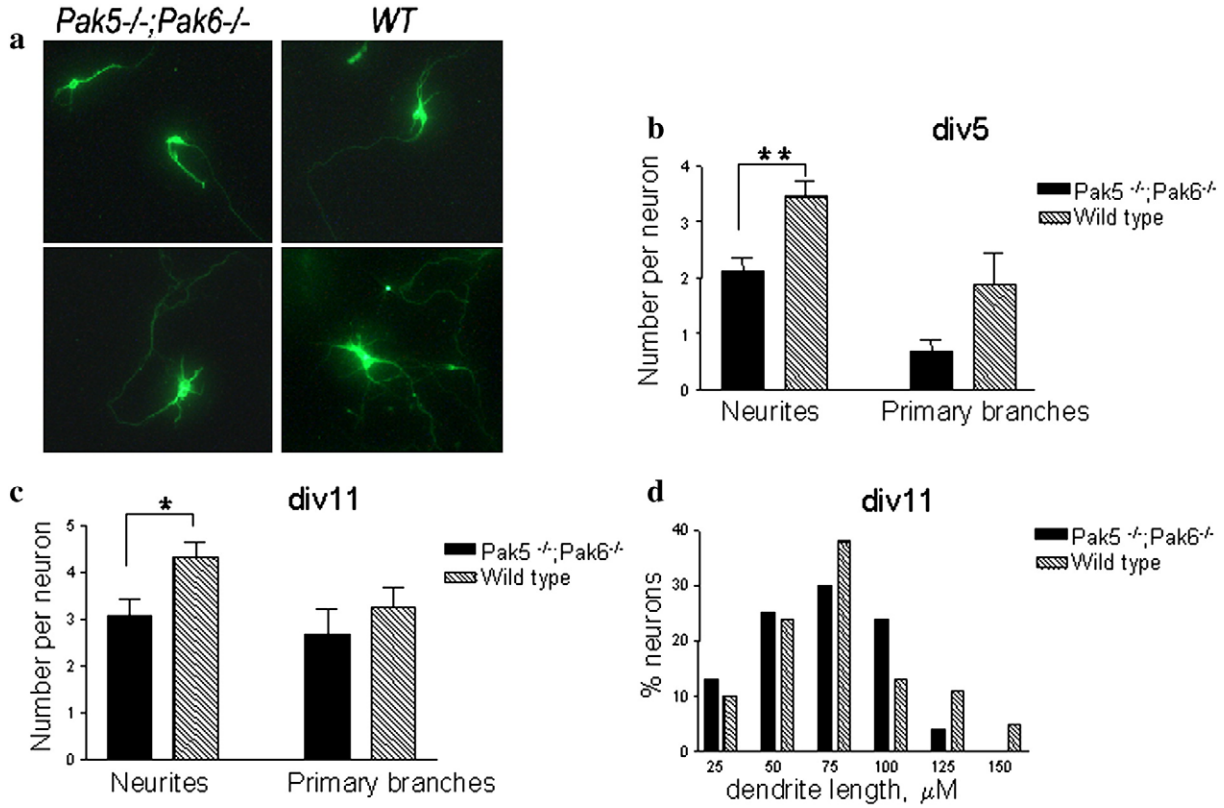

Fig. 10. Pak5$^{-/-}$;Pak6$^{-/-}$ cortical neurons in culture have a decreased number of neuronal processes. E14.5 cortical neurons were dissociated, plated on poly-L-lysine coated plates and allowed to develop for 5 (div5) or 11 (div11) days in culture. Cells were then fixed and stained with β-tubulin III to reveal neuronal morphology. Neurons were analyzed based on the length and number of neuronal processes and on their branching pattern. (a) Morphology of wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ neurons at div5. (b, c) Neurons from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice have decreased number of neuronal processes and a comparable number of primary branches at div5 (b) and div11 (c). Data shown as mean±SEM. $^{**}p<0.01$, $^{*}p<0.05$; Student's *t* test. (d) Decreased number of longer length neuronal processes in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cortical neurons at div11.

trials mice learned the location of the safe chamber and now they must inhibit that response. The test provides a second measure of learning and the tendency of mice to perseverate. *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice performed worse than wild type mice when comparing the number of animals who learned to inhibit their response (Fig. 7c). The time it took to make the incorrect response was also lower in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice (Fig. 7d) indicating they repeatedly entered the shock chamber. Taken together these results suggest that *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice have a decreased learning ability and impaired memory function.

Anxiety levels are normal in the Pak5$^{-/-}$;*Pak6*$^{-/-}$ *mice*

The elevated plus maze test is based on the natural aversion in rodents for high and open spaces and is used as a measure of anxiety. There was a significant preference for closed arms rather than the open arms for both genotypes (Fig. 8). *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice entered the closed arms significantly fewer times compared to the wild type mice (Fig. 8), but they also entered the open arms at a lower rate. The *Pak6* knockout mice behaved similarly to the wild type mice and *Pak5* knockout mice showed slightly increased number of entrances to the closed arm as compared to wild type mice (18.2±0.86 versus 12.25±1.35, respectively). (See Supplemental Fig. 3). There was no significant difference observed in jump-offs from the elevated plus maze. Since the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice entered both closed and opened arms fewer times, these results support the idea that there is a difference in a motor activity, but do not reveal a significant difference in anxiety in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. The only suggestion that there may also be a difference in anxiety levels is the finding that there was a slight increase in the amount of defecation (fecal boli) by the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice compared with the wild type mice.

Pak5$^{-/-}$;*Pak6*$^{-/-}$ *mice have normal levels of sociability but decreased levels of aggressive behavior*

Two different tests, the social chamber and the resident–intruder test, were used to study sociability and aggressive behavior. Mice were given 3 sessions to explore an area that contained two identical cylinders. One cylinder contained an unfamiliar mouse and the other one was left empty. Both groups of mice showed strong interest in a new mouse as compared with the empty cylinder, thus demonstrating a high level of sociability (Fig. 9a). On average, *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice had a slightly decreased number of contacts with both the occupied cylinder (110±23 contacts for *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice versus 174±19 for wild type mice) and the empty cylinder (41±2.6 contacts for *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice versus 58±3.8 contacts for wild type mice). However, these differences were not statistically significant, and may be due to a locomotor deficit rather than a difference in sociability.

The resident–intruder test was used to study aggression. In this test the male "resident" mice were housed individually for two weeks before the procedure, and the male "intruder mice" were housed in cages of five mice per cage. An intruder mouse was then placed in the cage with a resident mouse thus providing territory defense. The male *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice demonstrated a significantly lower number of bite attacks compared to wild type male mice (Fig. 9b). Interestingly, even the single knockout mice showed a decrease in aggression, however only the difference in aggression between *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ and wild type mice was statistically significant (see Supplemental Fig. 4). The total number of aggressive *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice was much lower than the number of aggressive wild type mice (trial 1: 1 in 11 *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice versus 3 in 10 wild type mice; trial 2: 1 in 11 *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice versus 5 in 10 wild type mice; trial 3: 2 in 11 *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice versus 5 in 10 wild type mice). The latency to attack was also

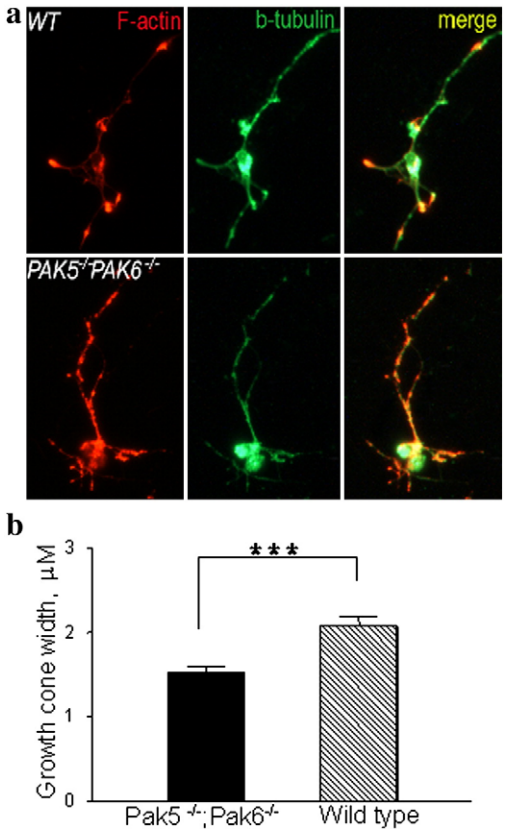

Fig. 11. Abnormal filopodia in Pak5$^{-/-}$;Pak6$^{-/-}$ cortical neurons. (a) Cortical neurons were fixed and stained with Alexa™555 Phalloidin to visualize F-actin, together with β-tubulin III to reveal neuronal morphology. Neurons from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice have significantly smaller growth cones. (b) Average width of the growth cone in wild type and Pak5$^{-/-}$;Pak6$^{-/-}$ neurons. Data shown as mean ± SEM. ***$p<0.001$, Student's *t* test.

examined. The time it took for the mice to initiate an attack was measured and averaged for each condition. The average attack latency times was significantly higher for *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice only in the third trial (Fig. 9c: 21.6 ± 5.4 s wild type mice versus 3.69 ± 0.9 s *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice). Aggressive behavior of wild type resident mice was comparable to behavior of the intruder mice (Fig. 9d) towards resident mice of any genotype. These results indicate that the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice display a lower level of aggression.

Serotonin and dopamine levels are normal in the double knockout mice

In mice, the neurotransmitters dopamine and serotonin can specifically affect aggressive behaviors and short- and long-term memory. We therefore investigated whether altered levels of monoamines could contribute to some of the social, cognitive, and motor deficits observed in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Levels of serotonin and dopamine and their metabolites were measured using HPLC-electrochemical detection in adult 30 and 120 days old male mice. Analysis of frontal cortex, hippocampus, striatum, hypothalamus and brain stem revealed no significant alterations between genotypes in the levels of serotonin, dopamine, or their metabolites (Table 1). These results indicate that the differences we see in aggression and other behaviors are most likely not due to differences in neurotransmitter levels.

Deficits in cortical cultures from Pak5$^{-/-}$;*Pak6*$^{-/-}$ *mice*

To gain insight into mechanisms behind the deficits in double knockout mice, we isolated and cultured cortical neurons from wild type and *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Neurons from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice were viable, attached well to the substrate and did not show any signs of degeneration or early death in culture (Fig. 10a). However, upon more detailed examination, several differences were revealed. Both young — day 5 *in vitro* (div5) and more mature (div11) *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ neurons had significantly lower numbers of neuronal processes (neurites) developed on the cell body. Number of neurites at div5 in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cells was 2.13 ± 0.23 compare to 3.44 ± 0.29 neurites in wild type cells, at div11 these numbers were 3.06 ± 0.36 versus 4.31 ± 0.33 mutant and wild type cells respectively (Figs. 10b, c). Interestingly, although at div5 the number of primary branches was slightly lower in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cortical neurons (Fig. 10b, $p>0.05$, t test) by div11 neurons of both genotypes developed comparable numbers of primary branches (Fig. 10c). Neuronal processes in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ were slightly shorter in double knockout neurons as compared with wild type neurons (Fig. 10d). Number and length as well as appearance of cells and neuritis in single knockout neuronal cultures were comparable to wild type cells (Supplemental Fig. 5). Interestingly, *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ neurons also revealed dramatic differences in the appearance of growth cones when stained with the F-actin marker phalloidin. Developing neurons from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice had very small growth cones, which were located not only on the tip of growing neuritis but could also be seen along the shafts of neuronal processes (Fig. 11a). When we compared the width of growth cones in cultured cortical neurons we found it significantly decreased in neurons from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice (growth cone width: wild type neurons 2.08 ± 0.10 µM versus 1.52 ± 0.05 µM in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ neurons; ***$p<0.001$) (Fig. 11b). In summary, there were differences in number and length of neuronal processes as well as abnormal growth cones in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ neurons, which could potentially account for neurological deficits found in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice.

Discussion

PAK4, PAK5, and PAK6 are members of the group B family of PAKs (Jaffer and Chernoff, 2002). *Pak4* knockout mice are embryonic lethal, while *Pak5* knockout mice have no visible phenotype (Li and Minden, 2003; Qu et al., 2003). The aim of this study was to investigate the biological and developmental roles of PAK6, and to determine whether PAK5 and PAK6 play important roles in locomotion and behavior. To achieve this we generated and characterized *Pak6* knockout mice (*Pak6*$^{-/-}$) and bred them to *Pak5*$^{-/-}$ mice to generate double knockout mice (*Pak5*$^{-/-}$;*Pak6*$^{-/-}$). In addition to generating knockout mice, we also carried out a detailed study of the expression patterns of the group B PAKs. We found that PAK5 and PAK6 proteins have similar expression patterns and are highly expressed in the brain. Their overlapping expression patterns suggest that they may have functional redundancy, and we therefore focused our studies on the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice were viable and fertile and did not have any obvious physical abnormalities. To determine the extent to which PAK5 and PAK6 are involved in control of the brain function, we analyzed the behavior of the mice. We found that *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice have deficits in locomotor activity as well as learning and memory. A role for PAK5 and PAK6 in locomotion is supported by the fact that *Pak4*, a group B *Pak*, is important in motor neuron development (Qu et al., 2003). A role in learning and formation of the memory is strongly supported by the finding that there is high expression of both PAK5 and PAK6 in the cortex, hippocampus and striatum, which are structures that are critically involved in cognitive functions.

Pak5$^{-/-}$;*Pak6*$^{-/-}$ mice clearly showed strong deficits in locomotion, as assessed by the open field test. The difference in horizontal activity persisted during the test suggesting that mice, regardless of the genotype, had similar habituation patterns and that the knockout mice are indeed impaired in locomotion. We also saw a decrease in aggressive behavior in the knockout mice, without a corresponding lack of sociability or interest in a novel mouse. This could also be related to a decrease in general activity of the mice. As mentioned

above, *Pak4* knockout mice were shown to have deficits in motor neuron development (Qu et al., 2003). Although motor neuron progenitors appeared normal, there are fewer than normal differentiated motor neurons, and those that do develop fail to migrate to their correct locations in the *Pak4* knockout mice (Qu et al., 2003). One possible explanation is the role for *Pak4* in regulation of the cytoskeleton and in filopodia formation, which is crucial for normal differentiation of neurons. Cultured cortical neurons from *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice showed abnormally small growth cones, possibly as a result of defects in cytoskeletal dynamics. Importantly, *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cortical neurons in culture developed significantly decreased number of neuronal processes and length of these processes was shorter. These deficits *in vivo* should critically affect proper neuronal contacts and wiring of the nervous system and lead to functional abnormalities. Decreased motor function in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice could result from decreased number of proper neuronal contacts in motor neurons. Alternatively, decreased synaptic strength due to changes in cytoskeletal organization could also affect motor control in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Our current results are consistent with a general role for the group B *Paks* in motor neuron development. It should be noted that *Pak5* and *Pak6* are expressed later during development compared with *Pak4*, which is expressed at high levels primarily during embryogenesis. This would support the idea that *Pak4* has a role in early differentiation of motor neurons, while *Pak5* and *Pak6* have a role later in their development or maintenance. Interestingly, no locomotor deficit was found in transgenic mice containing a dominant-negative mutant of the group A *Paks* (Hayashi et al., 2004), suggesting that a role in motor neuron development could be unique to the group B *Paks*. It should also be noted that the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice weigh slightly more than the wild type mice, and this could also contribute to the decrease in locomotor activity. However, the increase in weight is quite small compared with the decrease in activity, and it is difficult to differentiate between the increased weight being a cause or an effect of locomotor activity, or whether these two phenotypes are related at all.

In addition to deficits in locomotion, *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice also clearly showed significant impairment in learning. Learning abilities and memory function in the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice were assessed in the T-maze. *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice had delays in active avoidance learning as manifested by a decreased number of successful avoidances of the shock chamber and increased avoidance latency. *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice also exhibited a deficit in passive avoidance where they had to learn to inhibit a previously learned response. While the T-maze is a test used to assess learning and memory, several other factors, including basal locomotor activity and sensitivity to the shock, could also contribute to performance on the T-maze test. The fact that *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice escaped from the "shock" chamber as fast as wild type mice, however, argues against decreased sensory function in these mice. With regard to motor activity, results from the open field test did reveal a significant decrease in locomotor activity in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Likewise, the hanging wire test also demonstrated decrease in motor function and muscle strength in the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. Although a decrease in motor activity could affect the results of the T-maze test, we believe that the results show an actual deficit in learning. In the passive avoidance procedure, the knockout mice actually engaged in the motor response more than the wild type mice. They could not learn to inhibit their response. In addition, *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice had the same escape latency as wild type mice. Taken together, these results suggest that a deficit in learning and memory is a significant factor in the performance in the T-maze, active and passive avoidance test.

The mechanism behind the behavioral changes in the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice needs to be explored further. Evidence from studies of different organisms ranging from invertebrates to humans indicates that the serotonin (5-hydroxytryptamine; 5-HT) system modulates short- and long-term memory (Liy-Salmeron and Meneses, 2007). However, we did not find changes in the levels of serotonin, dopamine or their metabolites in our study, which suggests that changes in learning and motor activity in the *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice are independent from neurotransmitters or might occur at the level of neurotransmitter uptake due to changes in synaptic density or structure. Another intriguing possibility is that deficits in learning and the formation of the memory might result from changes in postsynaptic strength due to changes in the morphology of cortical and hippocampal dendritic spines in the brains of *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice. This would be consistent with the role for other PAK proteins in the regulation of the neural cytoskeleton and the formation of dendritic spines (Hayashi et al., 2004, 2007; Meng et al., 2005). Decreased size and improper location of growth cones in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cortical neurons point to changes in the cytoskeletal dynamics — an essential aspect of neuronal structural plasticity and function of central nervous system. One more possibility is that axonal length and diameter are affected in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ mice and such changes could result in decreased conductivity, leading to deficits in learning and memory. This is strongly supported by our finding that *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cortical neurons develop less long neuronal processes. In fact, PAK5 was shown to play a positive role in guanine nucleotide exchange factor T (GEFT) induced neurite outgrowth in neuroblastoma cells (Bryan et al., 2004), and can promote neurites on its own when activated (Dan et al., 2002). The role for PAK6 in the promotion of neurite outgrowth has not yet been explored but given its similarity to PAK5 in both sequence and expression pattern, we predict that it may also have a key role in neuronal development. It is interesting that PAK5 and PAK6 seem to show complete redundancy in locomotion, but can also just partially compensate for each other in some functions. In learning tests and aggression tests, for example, single knockouts show some decrease in function, although double knockout mice demonstrate more profound and significant deficits. This suggests at least a partial overlap between the functions of PAK5 and PAK6.

Our results showing a link between PAK proteins in learning and memory as well as locomotion are consistent with recent studies in animals as well as in humans. Roles for PAK5 and PAK6 in learning and memory are congenial, for example, with the roles for Rho GTPases and PAK proteins in regulating the neuronal cytoskeleton (Luo, 2000; Luo et al., 1996; Tashiro et al., 2000; Threadgill et al., 1997). Experimental evidence indicates that the neuronal cytoskeleton regulates morphology and rearrangement of dendritic spines (Fukazawa et al., 2003; Zito et al., 2004), highly dynamic structures involved in formation of synaptic connections in neural networks. Rearrangement of synaptic connections is thought to be the basis for learning and formation of memory (Muller et al., 2000; Trachtenberg et al., 2002). The shapes of dendrites and dendritic spines are regulated by Rho GTPases and are affected in some types of mental retardation (Purpura, 1974). A deficit in activated Rho GTPases and their effector proteins has also been demonstrated in some types of mental retardation (Allen et al., 1998; Pasteris et al., 1994). A crucial role for Rho GTPases in cognitive function is also supported by the finding that activation of Rho GTPases in the brain leads to rearrangement of the cerebral actin cytoskeleton, enhanced neurotransmission, and improvement of learning and memory (Diana et al., 2007). Discovery of abnormal growth cones in *Pak5*$^{-/-}$;*Pak6*$^{-/-}$ cortical neuronal cultures pointed to possible changes in cytoskeletal dynamics, which can lead to formation of abnormal dendritic spines and result in impairment in synaptic contacts and further deficits in plasticity during learning.

Downstream effectors for Rho GTPases involved in regulation of cognitive function are only recently starting to being identified. Roles for PAK1 and PAK3 in this process were recently discovered. Abnormalities in synaptic plasticity and deficiencies in learning and memory were found in *Pak3* null mice (Meng et al., 2005). Significant alterations in the morphology of dendritic spines and synaptic structure were found in the brains of transgenic mice containing

dominant-negative *Pak* (Hayashi et al., 2004). In these mice the catalytic activity of the group A PAKs was inhibited in the postnatal forebrain, which led to changes in synaptic strength and deficits in consolidation of the memory. Interestingly, inhibition of the catalytic activity of the group A PAKs affected the morphology of cortical dendrites, while the dendritic spines in the hippocampus, which is involved in detection of new stimuli, were not affected (Hayashi et al., 2004). Pharmacological inhibition of PAK also causes memory impairment in mice (Zhao et al., 2007). A role for PAK proteins in learning and memory in humans is also suggested by the finding that the activity of PAK1 and PAK3 and the localization of phosphorylated PAK is altered in patients with Alzheimer's disease (Zhao et al., 2006). Mosaic deletion (20pter → p12.2) within the genomic region where human *Pak5* gene is located (20 p12.2) was found in a patient with moderate to severe mental retardation, autistic behavior patterns, and myoclonic epilepsy of early childhood (Sauter et al., 2003), which suggest that group B *Paks* are also closely tied to cognitive and behavior functions in humans.

PAK proteins may also be implicated in motor neuron development and function in humans. An interesting example is lateral sclerosis, a disease associated with motor neuron degeneration. Approximately 10% of patients with lateral sclerosis have a family history for the disease. Several loci were identified as being involved in inherited forms of the disease, although not all of the genes involved have been identified. Interestingly, human *Pak6* gene (15q15.1) is located within the locus ALS5 (15q15-q22) (Hentati et al., 1998) and it will be extremely interesting to study whether deletion of *Pak6* in humans is involved in inherited forms of lateral sclerosis.

In future studies it will be interesting to study the molecular mechanism by which *Pak5* and *Pak6* regulate learning, memory, and motor function in more detail. It will also be important to study *Pak5*/*Pak6* substrates that could mediate their functions. One possible candidate could be LIM Kinase (LIMK), a substrate for several PAKs, including PAK4 and PAK5 ((Dan et al., 2001; Edwards et al., 1999), and Minden unpublished results). A defect in LIMK1 is linked to Williams syndrome, a neurodevelopmental disorder characterized by a distinctive array of learning and behavioral disabilities (Frangiskakis et al., 1996; Morris and Mervis, 2000). Also of interest is the role for *Pak6* in binding and downregulating the androgen receptor (Lee et al., 2002; Schrantz et al., 2004; Yang et al., 2001). However, our results do not reflect an increased level of androgen receptor activity in the knockout mice, due to the finding that $Pak5^{-/-};Pak6^{-/-}$ male mice weighed more than wild type males and are less aggressive.

Our findings clearly demonstrated an essential role for group B PAKs, PAK5 and PAK6, in the regulation of behavior. Overall, it will be interesting to explore the relationship between the abnormalities observed in the double knockout mice and motor neuron diseases, such as Charcot-Marie-Tooth disease or lateral sclerosis, as well as with various forms of mental retardation (Mitchell and Borasio, 2007; Reilly, 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.07.006.

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