Identification of the genomic mutation in \(Eapha4^{rb-2J}/rb-2J\) mice


Abstract: The EphA4 receptor tyrosine kinase is involved in numerous cell-signalling activities during embryonic development. EphA4 has the ability to bind to both types of ephrin ligands, the ephrinA and ephrinB. The C57BL/6J-\(Eapha4rb-2J/GrsrJ\) strain, denoted \(Eapha4^{rb-2J}/rb-2J\), is a spontaneous mouse mutant that arose at The Jackson Laboratory. These mutants exhibited a synchronous hind limb locomotion defect or “hopping gait” phenotype, which is also characteristic of EphA4 null mice. Genetic complementation experiments suggested that \(Eapha4^{rb-2J}\) corresponds to an allele of EphA4, but details of the genomic defect in this mouse mutant are currently unavailable.

We found a single base-pair deletion in exon 9 resulting in a frame shift mutation that subsequently resulted in a premature stop codon. Analysis of the predicted structure of the truncated protein suggests that both the kinase and sterile \(\alpha\) motif (SAM) domains are absent. Definitive determination of genotype is needed for experimental studies of mice carrying the \(Eapha4^{rb-2J}\) allele, and we have also developed a method to ease detection of the mutation through RFLP. Eph-ephrin family members are reportedly expressed as numerous isoforms. Hence, delineation of the specific mutation in EphA4 in this strain is important for further functional studies, such as protein–protein interactions, immunostaining and gene compensatory studies, investigating the mechanism underlying the effects of altered function of Eph family of receptor tyrosine kinases on phenotype.

Key words: EphA4, hopping gait, spontaneous mutation, knockout mouse, rb-2J strain.

Résumé : Le récepteur à activité tyrosine kinase EphA4 est impliqué dans de nombreuses activités de signalisation cellulaire au cours du développement embryonnaire. L’EphA4 a la capacité de lier les deux types d’éphrines, les éphrinesA et éphrinesB. La souche C57BL/6J-\(Eapha4rb-2J/GrsrJ\), connue en génétique sous le nom de \(Eapha4^{rb-2J}/rb-2J\), est un mutant spontané survenu au Jackson Laboratory. Ces mutants présentent un défaut de motricité synchrone des membres postérieurs, une démarche sautillante, qui est également caractéristique du mutant nul EphA4. Des expériences de complémentation génétique ont suggéré que l’\(Eapha4^{rb-2J}\) serait un allèle d’EphA4, mais les détails de l’altération génomique chez ce mutant demeurent inconnus. Les auteurs ont trouvé une délétion d’un seul nucléotide dans l’exon 9 qui entraine un changement de cadre de lecture et produit un codon stop prématuré. L’analyse de la structure prédite de la protéine tronquée suggère que deux domaines importants seraient perdus, le domaine kinase et le domaine SAM (« sterile \(\alpha\) motif »). Une identification certaine du génotype est également nécessaire pour les expériences impliquant l’allèle \(Eapha4^{rb-2J}\), et les auteurs ont mis au point une méthode pour...
détecter la mutation par le biais d’un RFLP. Différents isoformes sont rapportés pour les membres de la famille Eph-éphrine. Ainsi, l’identification de la mutation spécifique dans EphA4 dans cette souche est importante pour de futures analyses fonctionnelles (interactions protéine–protéine, immuno-localisation et études de compensation génétique) visant à déterminer le mécanisme reliant les effets d’une fonction altérée de la famille de récepteurs à activité tyrosine kinase Eph au phénotype. [Traduit par la Rédaction]

Mots-clés : EphA4, démarche sautillante, mutation spontanée, souris knock-out, souche rb-2J.

Introduction

Erythropoietin hepatocellular carcinoma receptor tyrosine kinases, more commonly known as the Ephs, are members of the largest family of receptor tyrosine kinases (RTKs) that characteristically bind to their ligand ephrins. The mammalian and chick Ephs are divided into two groups based on sequence homologies and affinity for ephrins, the A-type (EphA1–EphA10) and the B-type (EphB1–EphB6) (Pasquale 2008). EphrinAs are known to bind to EphA receptors and ephrinBs bind to EphBs. EphA4 interacts with both ephrinAs and ephrinBs (ephrinB2 and ephrinB3) and ephrinA5 binds to Ephb2 (Himanen et al. 2004; Pasquale 2004). Eph and ephrin signalling play many important roles including remodelling of blood vessels and formation of tissue boundary (Pasquale 2005, 2008; Wilkinson 2015). EphA4 has been shown to be involved in cell signalling activities in numerous contexts including axon guidance and development of central nervous system vasculature (Dottori et al. 1998; Kullander et al. 2001).

During embryonic development, EphA4 is expressed at the tips of the closing spinal neural folds, and in the developing forebrain, hindbrain, and mesoderm (Abdul-Aziz et al. 2009; Nieto et al. 1992). In adult mice, EphA4 is highly expressed in the jejunum (Islam et al. 2010) and the brain, mainly in the hippocampus (Greffrath et al. 2002; Grunwald et al. 2004; Kullander et al. 2001). Targeted mouse knockouts of the EphA4 gene display locomotor abnormalities of the hind limb resulting in rabbit-like hopping movements (Coonan et al. 2001; Herrmann et al. 2010; Kullander et al. 2001, 2003; Nieto et al. 1992) or clubfoot of the hind limb (Helmbacher et al. 2000). Interestingly, inactivation of the EphA4 function, which causes the lack of axonal guidance, was reported to promote axonal regeneration and improve functional recovery of a central nervous system injury (Goldshmit et al. 2011).

Two spontaneous mutations of the EphA4 gene, Epha4^{rb-2J} (Eph receptor A4; rabbit) and Epha4^{rb-2J/rb-2J} (Eph receptor A4; rabbit 2 Jackson), were reported as spontaneous recessive mutants within the C57BL/6J background at The Jackson Laboratory. However, the exact genomic location of the mutated alleles has not been ascertained. Since the phenotype observed in the Epha4^{rb-2J/rb-2J} mice was very similar to the targeted knockouts of the EphA4 gene, complementarity testing was performed by mating a Epha4Gt(pGT1TM)38Wcs/+ female (provided by Tessier-Lavigne Laboratory of Stanford University) to an Epha4^{rb-2J/+}

male. Among the progeny of this cross, three pups (one female and two males) out of seven exhibited hopping gait phenotype without leaning. The information on the Epha4^{rb-2J} and Epha4^{rb-2J/rb-2J} are from the Mouse Mutant Resource Web Site, The Jackson Laboratory, Bar Harbour, Maine (http://mousemutant.jax.org/, accessed 9 October 2010). This finding of genetic complementation strongly suggested that the rb-2J mutation lies within the EphA4 gene.

The aim of this study is to understand the nature of the Epha4^{rb-2J/rb-2J} mutation and the extent of the mutation in the protein. Therefore it was necessary to define the mutation and determine the exact genotypes of the mice. We also reviewed the impact of the mutation on EphA4 isoforms and other reported EphA4 mutants to better understand the diversity of the EphA4 protein. In addition, we describe a rapid, simple assay for genotyping the C57BL/6J-Epha4rb-2J/GrsrJ mice, which was not previously available and will be invaluable to other groups intending to work with these mice. Therefore, we have addressed the knowledge gap for the C57BL/6J-Epha4rb-2J/GrsrJ mouse in this study.

Materials and methods

The Epha4^{rb-2J/rb-2J} strain (003129) was obtained from The Jackson Laboratory and the colony maintained at the Universiti Kebangsaan Malaysia’s Animal Biosafety Level 2 laboratory. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Malaya (approval number PAR/20/09/2011/NMAA).

Epha4^{rb-2J/rb-2J} Knockout mouse and dissection of the hippocampus

As the hopping gait phenotype is only evident in mice at 3 weeks old, mutational analysis was first determined using samples from adult mice to correlate the genotype with an affected phenotype. In adult mice, EphA4 expression is abundant in the hippocampus (Liebl et al. 2003; Murai et al. 2003), therefore hippocampi of two aged matched control and Epha4^{rb-2J/rb-2J} mice were isolated (modified protocol) (Fuller and Dailey 2007). The mice were euthanized by cervical dislocation. After the midline incision from the foramen magnum was made up to the level of eye sockets, the skull flaps were tilted to break it off using forceps, and the brain was lifted out of the skull vault gently using curved narrow patterned forceps. In the clean petri dish, the brain was cut into half at the midline, and the hemisphere of the brain was  

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RNA isolation

RNA isolation was carried out by homogenisation in 1 mL of TRIzol (Invitrogen, Carlsbad, Calif., USA) to 50 mg of hippocampi tissue, followed by the addition of 0.2 mL of chloroform according to the manufacturer’s instruction. After separation by cold centrifugation, the aqueous phase was placed in a new tube before addition of 0.5 mL of isopropanol and subsequently centrifuged. The pellet was washed with 75% ethanol and left to air-dry for 5–10 min. The pellet was then re-suspended in distilled, deionised water.

**Mutational screening of EphA4**

Seven sets of primers, which amplify overlapping regions of EphA4 cDNA, were designed to allow sequencing of the entire coding region (accession number: NM_007936). After the discovery of location of the putative mutation in EphA4, primers for genomic DNA were also designed. All primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Primer sequences with overlapping region of EphA4 cDNA were as follows: set 1 flanking exon 1 to 3 (668 bp), 5′-CACCTCTTGCCAATGCTTT-3′ and 5′-CTTTCTCACGATTGGTGCT-3′; set 2 flanking exon 3 to 5 (700 bp), 5′-GACATTGGTGACCCATTAT-3′ and 5′-TCCACTTACACCCACCAGAGA-3′; set 3 flanking exon 5 to 8 (588 bp), 5′-GCGGTCGAGCAATTCCTTA-3′ and 5′-ACCTCCAGTCTGGCTGTC-3′; set 4 flanking exon 7 to 11 (499 bp), 5′-CTGACATTCTATGTTTTCAG-3′ and 5′-GAGACCTCTGAGTTGAGGCC-3′; set 5 flanking exon 10 to 14 (578 bp), 5′-CAGATGACATCCGAAA-3′ and 5′-CTAGACGGATGATGTCTGGAG-3′; set 6 flanking exon 13 to 16 (515 bp), 5′-CGAAGCGCAATCTACACTACC-3′ and 5′-TAGAAGCCGCTGTTCCATG-3′; and set 7 flanking exon 15 to coding region exon 17 (471 bp), 5′-CTCCCCCTGAAATCTCTGCATG-3′ and 5′-ATCAGAATATAACCCTGGAGCCA-3′.

RT-PCR was performed using Transcriptor One-Step RT-PCR kit (Roche Diagnostics, Mannheim, Germany). The transcription enzyme mix contains transcriptase, expand system, and protector RNAse inhibitor. The 1X reaction buffer includes Tris, MgCl₂, 1.5 mmol/L dNTPs and additives for hot start PCR. We generated cDNA and amplified 50 ng of total RNA in 50 μL of reaction mixture consisting of 0.4 μmol/L of each primer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (Pgk1) mRNA were used as internal controls. The reverse transcription and amplification condition was set as follows: cDNA generation at 50 °C for 30 min, and 94 °C for 7 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at optimised temperature for 30 s, extension at 68 °C for 1 min, with final extension at 68 °C for 7 min. Resulting PCR products were analysed by electrophoresis on 1% agarose gels containing ethidium bromide.

RT-PCR products from EphA4+/+, EphA4+/−, and EphA4−/− samples were purified using QIAquick PCR purification kit or QIAquick gel extraction kit (Qiagen, Valencia, Calif., USA). All the purification steps were according to the manufacturer’s directions. Subsequently, 10 μL of 30 ng/μL single band PCR products were sent for Sanger sequencing through a commercial company.

**Genomic DNA isolation and PCR**

While the mutational screening was performed on the coding transcripts, to enable ease of genotyping from the genomic DNA, we developed a method to genotype the mutation from genomic DNA. Tail or ear clips of adult mice were obtained and DNA extraction for genotyping was performed as previously described (Sambrook et al. 1989). The tissues were lysed in NET buffer containing 20 mmol/L Tris-Cl pH 8.0 (Sigma), 5 mmol/L EDTA pH 8.0 (Sigma), 400 mmol/L NaCl (Sigma), 1% (w/v) SDS (Sigma), and sterilised by filtration through 0.45 μm nitrocellulose filter before the addition of 20 mg/mL Protease K (Sigma). The tissues were incubated at 55 °C until the tissue was completely lysed. The DNA was isolated by using 1:1 of phenol solution (Sigma), followed by 2:1 of absolute ethanol precipitation, and 70% ethanol washing step. After left to air-dry, the pellet was re-suspended in distilled, deionised water.

The genomic DNA amplification of samples were performed with 0.6 mmol/L of EphA4 primers spanning the mutation (accession number: NC_000067.6; forward P1: 5′-GAGACCTCTGAGTTGAGGCC-3′ and reverse P2: 5′-CAAGGGATATTAACCAACACTTC-3′) in a 50 μL total reaction of DreamTaq Green DNA polymerase (ThermoScientific), 1× buffer DreamTaq Green buffer including 2 mmol/L MgCl₂, and 0.2 mmol/L dNTP. The amplification condition was set as follows: 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min, with final extension at 72 °C for 10 min. The expected size of amplicon was 258 bp. Subsequently, 40 μL of 30 ng/μL single band PCR products were sent for DNA purification and Sanger sequencing through a commercial company.

**PCR-RFLP (restriction fragment length polymorphism)**

We employed PCR-RFLP to rapidly genotype the novel mutation in more samples. New primers were designed based on the EphA4−/−/+ exon 9 nucleotide sequence (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to introduce mutations into the amplified DNA at nucleotide 1799 and 1800 (AA→TC) of the gene to generate an XhoI recognition site CTCGAG in mutant samples. The forward primer sequence (P3) was 5′-TGAGAACGGTTTTCTGGAGAGAGAATCGTCTAGAGG-3′ (the altered sequence was underlined) and
reverse primer sequence (P2) was 5′-CACAGGCAATATT AACCAACACTTC-3′. We amplified 50 ng of genomic DNA in a 20 µL of reaction mixture consisting of 0.5 µmol/L of each primer and 1× LightCycler® 480 Probe Master containing FastStart Taq DNA Polymerase, dNTP mix, and 6.4 mmol/L MgCl₂ (Roche Diagnostics, Mannheim, Germany). The amplification condition was set as follows: 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 53 °C for 30 s, extension at 72 °C for 10 s, with final extension at 72 °C for 7 min. Approximately 15 µL of amplicon was digested with 20U of XhoI (New England Biolabs, Ipswich, Mass., USA) according to manufacturer’s protocol followed by separation on 4% agarose gel. The fragments generated were a single 149 bp for the homozygote mutant, a single 166 bp band for wildtype, and two bands of 149 and 166 bp for heterozygous profiles.

**Western blot analysis**

To determine the effect of the mutation, immunoblot analysis was performed using anti-EphA4 (EphA4 Antibody S-20; sc-921) and anti-GAPDH (Santa Cruz Biotechnology) on wildtype, heterozygous, and mutant protein samples isolated from the hippocampi of 31-day-old mice. Protein amounting to 25 µg isolated from the hippocampi was mixed with 2 µL of 1 mol/L DTT in a final volume of 20 µL. Protein samples were heated to 100 °C for 10 min then immediately placed on ice prior to gel loading. Life Technologies Xcel Surelock Mini Cell system was used with a 4%–12% denaturing precast protein gel according to the manufacturers guidelines. Samples were run at 200 V for 1 h. Protein was then transferred using Life Technologies Xcel Mini Cell transfer system per manufacturers guidelines. Protein was transferred at 35 V for 3 h with transfer apparatus submerged in ice bucket. Blot was then blocked with 5% milk in TBS-T

**Table 1. EphA4<sup>rb-2J/rb-2J</sup> mutation lies within a conserved region.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphA4 wildtype (mouse)</td>
<td>AKQEADEEKHL...</td>
</tr>
<tr>
<td>EphA4 mutant (mouse)</td>
<td>AKQEQMKRNI-stop</td>
</tr>
<tr>
<td>Human</td>
<td>AKQEADEEKHL......</td>
</tr>
<tr>
<td>Orangutan</td>
<td>AKQEADEEKHL...</td>
</tr>
<tr>
<td>Xenopus</td>
<td>AKQEADEEKHL......</td>
</tr>
<tr>
<td>Pig</td>
<td>AKQEADEEKHL......</td>
</tr>
<tr>
<td>Rat</td>
<td>AKQEADEEKHL......</td>
</tr>
<tr>
<td>Chick</td>
<td>AKQEADEEKHL......</td>
</tr>
</tbody>
</table>

**Note:** Alignment of EphA4<sup>rb-2J/rb-2J</sup> protein sequences of different organisms including human, rat, and Xenopus revealed that the spontaneous mutation was in a conserved region.
buffer (50 mmol/L Tris Base, 150 mmol/L NaCl, 0.1% Tween 20, pH 7.5) for 1 h. After blocking, anti-EphA4 primary antibody was diluted to 1:100 in 5% milk in TBS-T buffer and incubated with blot overnight at 4 °C. Anti-GAPDH primary antibody was added after 24 h incubation with anti-EphA4 at a dilution of 1:50 000 and was incubated for an additional 1 h at 4 °C. Blot was washed five times with TBS-T buffer for 5 min per wash with agitation. Secondary antibody (Life Technologies; A24531) was diluted 1:10 000 in 5% milk in TBS-T buffer and incubated with blot for 1 h at room temperature. Blot was further washed five times with TBS-T buffer for 5 min per wash with agitation. Secondary antibody was detected by mixing equal volumes of ECL reagents (Pierce; 34077) and exposing the blot to the mixed ECL reagents for 5 min. The blot was imaged on a BioRad chemidoc station.

Fig. 2. A schematic representation of Eph receptor, EphA4+/+, and EphA4rb-2j/rb-2j structure. (A) A general structure of an Eph receptor. (B) The predicted structures using Simple Modular Architecture Research Tool, SMART, for EphA4+/+. (C) SMART structure of EphA4 isoform at 104 kDa lacking kinase domain but possessing SAM domain. (D) SMART structure of EphA4 isoform at 63 kDa, which is truncated downstream of the juxtamembrane domain. (E) The EphA4rb-2j/rb-2j structure as predicted by SMART showing truncation downstream of the juxtamembrane domain, with consequent absence of the kinase and SAM domains.

Fig. 3. EphA4 protein expression. Western blot analysis of EphA4 immunostaining showed that the EphA4 mutant mice lacked the expression of the 110 kDa full-length protein but detection of a second band similar to EphA4 isoform at 104 kDa (UniProt). The 110 kDa full-length protein was detected in the EphA4 wildtype profile and heterozygous mice.

Fig. 4. A representative PCR-RFLP analysis of the EphA4rb-2j/rb-2j mice genotypes. PCR-RFLP samples were resolved in 4% agarose gel. Lanes 1–3 represent the wildtype profiles (166 bp band only), lanes 4–6 represent the heterozygote profiles (149 and 166 bp bands), lanes 7–9 represent the homozygote mutant profiles (149 bp band only), and lane 10 represents the 50 bp ladder.
Table 2. Phenotypes of EphA4 mutation mice based on position of mutation.

<table>
<thead>
<tr>
<th>Position of mutation</th>
<th>EphA4 mutation</th>
<th>Type of knockout and method</th>
<th>Phenotype and anatomical defects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 knockout and exon 3 frame shift mutation</td>
<td>EphA4&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Replacement vector; lac-Z reporter fusion</td>
<td>Hind limb phenotype (“club foot”) high penetrance</td>
<td>Helmbacher et al. 2000</td>
</tr>
<tr>
<td>Ligand binding domain (exon 3)</td>
<td>EphA4&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Gene replacement pgk-neo</td>
<td>Kangaroo-like (ROO) hopping gait</td>
<td>Dottori et al. 1998; Coonan et al. 2001</td>
</tr>
<tr>
<td>Extracellular region (exon 3)</td>
<td>EphA4&lt;sup&gt;EGFP&lt;/sup&gt;</td>
<td>Insertion of EGFP (reporter)</td>
<td>Hopping gait</td>
<td>Grunwald et al. 2004</td>
</tr>
<tr>
<td>Deletion of exon 3</td>
<td>EphA4 conditional; EphA4&lt;sup&gt;Flox&lt;/sup&gt;</td>
<td>Knock-in mCFP reporter gene; targeted (floxed/Frt)</td>
<td>Hind limb hopping gait</td>
<td>Herrmann et al. 2010</td>
</tr>
<tr>
<td>Deletion of exon 3</td>
<td>EphA4&lt;sup&gt;null&lt;/sup&gt;</td>
<td>Knock-in mCFP reporter gene</td>
<td>Hind limb hopping gait</td>
<td>Herrmann et al. 2010</td>
</tr>
<tr>
<td>Entire intracellular is missing</td>
<td>EphA4&lt;sup&gt;EGFP&lt;/sup&gt;</td>
<td>Entire intracellular part was replaced by green fluorescent protein (GFP)</td>
<td>Hopping gait</td>
<td>Egea et al. 2005</td>
</tr>
<tr>
<td>Fibronectin domain (aberrant amino acids after position 439, and truncation at position 442. T&gt;C intron 6 at position 113891 mutation (NC_000067). Possibility, splicing of exon 6 and frameshift that create stop codon early in exon 7</td>
<td>Frog</td>
<td>Chemical induced (ENU)</td>
<td>Hopping gait</td>
<td>Milstein et al. 2010</td>
</tr>
<tr>
<td>Juxtamembrane domain (Y596E and Y602E)</td>
<td>EphA4&lt;sup&gt;EIE&lt;/sup&gt;</td>
<td>Knock-in strategy (similar to Kullander et al. 2001), replacement vector—glutamic acid residues replace juxtamembrane tyrosines</td>
<td>Normal alternating gait. No discernible phenotype Abnormal thalamocortical topography; and partly defective central pattern generator (CPG) rhythmicity</td>
<td>Egea et al. 2005</td>
</tr>
<tr>
<td>Juxtamembrane domain (Y596F and Y604F)</td>
<td>EphA4&lt;sup&gt;EIE&lt;/sup&gt;</td>
<td>Knock-in strategy (targeting vectors)</td>
<td>Hopping gait</td>
<td>Kullander et al. 2001</td>
</tr>
<tr>
<td>Kinase domain and truncated at E582. Deletion adenine at 1802 (NM_007936)</td>
<td>EphA4&lt;sup&gt;b-2J&lt;/sup&gt;</td>
<td>Spontaneous mutation</td>
<td>Hopping gait</td>
<td>This study; Cook et al. 2004</td>
</tr>
<tr>
<td>Kinase domain (K653M)</td>
<td>EphA4&lt;sup&gt;KD&lt;/sup&gt;</td>
<td>EphA4 Knock-in strategy (targeting vectors); kinase dead</td>
<td>Hopping gait</td>
<td>Kullander et al. 2001</td>
</tr>
<tr>
<td>SAM domain (905–974 amino acids deletion); leaving 12 last amino acids residues intact</td>
<td>EphA4&lt;sup&gt;ΔSAM&lt;/sup&gt;</td>
<td>EphA4 Knock-in strategy (targeting vectors)</td>
<td>Normal alternating gait</td>
<td>Kullander et al. 2001</td>
</tr>
</tbody>
</table>
Results

The Epha4\(^{rb-2J/rb-2J}\) mouse is commercially available from The Jackson Laboratory. However, the mutation was not known and there is no clear protocol on how to genotype these mice. Knowing the exact genotype is crucial when investigating the effect of the loss of the gene in the affected mice. Therefore, in this study we determined the precise mutation in the Epha4\(^{rb-2J/rb-2J}\) strain, confirming that EphA4 loss of function is the cause of the phenotype in this strain and facilitating further experimental studies.

A single nucleotide deletion in Epha4\(^{rb-2J/rb-2J}\) results in a truncated EphA4 protein

The EphA4 gene is 6328 bp in size located on mouse chromosome 1 (Ensembl; release 80, May 2015). The sequences of the translated regions, contained within exon 1-17, of the Epha4\(^{rb-2J/rb-2J}\) strain were compared against the annotated EphA4 gene deposited in GenBank and with control C57BL/6J mice (accession number: NM_007936). A single nucleotide deletion (del1802) was located in exon 9 at 77,390,062 on mouse chromosome 1 and confirmed by sequencing of genomic DNA. This deletion is predicted to result in a frame shift and creation of a premature stop codon (Fig. 1A). The resultant protein is therefore predicted to consist of the wildtype sequence up to amino acid 582 (E582), followed by a series of six altered amino acids and a stop codon (Fig. 1B). The mutation lies within a conserved protein region in several organisms including human, orang-utan, frog, pig, rat, and chick (Table 1).

This truncated protein lacks a further 390 amino acids compared to the full-length wildtype protein, which has 986 amino acids (Fig. 1B). The protein structure encoded by Epha4\(^{rb-2J/rb-2J}\) was predicted using Simple Modular Architecture Research Tool, SMART (http://smart.embl-heidelberg.de). The predicted structure of the truncated proteins lacks the kinase and sterile alpha motif (SAM) domains (Fig. 2E).

Western blot analysis (Fig. 3) revealed an intact 110 kDa band in samples from both wildtype and heterozygous mice. This band was absent in the homozygous mutant mice. A second band at 104 kDa was detected in samples from all genotypes. We hypothesise that the lower molecular band may correspond to an alternate uncharacterised isoform. There are no reported isoforms of EphA4 with only exon 9 (containing the truncating mutation) spliced out.

An RFLP-PCR assay was developed to rapidly genotype the mice

In addition to providing a means to identify mice carrying the deletion on exon 9, we analysed C57BL/6J-Epha4\(^{rb-2J/rb-2J}\)/GrsrJ mouse colony with and without hopping gait characteristics using an inexpensive PCR-RFLP analysis (see Materials and methods). The analysis showed Epha4\(^{+/+}\) mice with wildtype profile (a single 166 bp band), Epha4\(^{rb-2J/+}\) mice with heterozygous profile (two bands at 149 and
Table 3. Isomers of mouse EphA4 protein.

<table>
<thead>
<tr>
<th>UniProt ID*</th>
<th>Size of transcript (bp)†</th>
<th>Protein (aa, amino acids)</th>
<th>Size (Da)</th>
<th>Aligned to Q03137 (position)</th>
<th>Domain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q03137 Long isoform (Experimental evidence at protein level)</td>
<td>6328</td>
<td>986</td>
<td>109 814</td>
<td>1–986 aa</td>
<td>Ligand binding domain (30–209 aa), cysteine rich domain (191–325 aa), fibronectin type III domain (328–439 aa and 440–537 aa), juxtamembrane domain, protein kinase domain (621–882 aa), SAM domain (911–975 aa), PDZ-binding motif (984–986 aa)‡</td>
</tr>
<tr>
<td>Q03137-2 Shorter isoform (No experimental confirmation available)</td>
<td>No information</td>
<td>936</td>
<td>103 984</td>
<td>Missing 783–832 aa (missing protein kinase domain). Missing whole exon 14</td>
<td>Ligand binding domain, fibronectin type III domain, juxtamembrane domain, SAM domain</td>
</tr>
<tr>
<td>Q3V1W9 Short isoform (Experimental evidence at transcript level)</td>
<td>No information</td>
<td>572</td>
<td>63 034</td>
<td>Missing whole exon 9–17 (missing juxtamembrane domain, kinase domain, SAM domain, and PDZ-binding motif)</td>
<td>Ligand binding domain, fibronectin type III domain</td>
</tr>
<tr>
<td>A0A087WRH4 (Experimental evidence at protein level)</td>
<td>614</td>
<td>117</td>
<td>13 206</td>
<td>833–949 aa (whole exon 15 and 16)</td>
<td>SAM domain</td>
</tr>
<tr>
<td>A0A087WQW6 (Experimental evidence at protein level)</td>
<td>584</td>
<td>177</td>
<td>19 075</td>
<td>328–473 aa (exon 5 and 6)</td>
<td>Fibronectin type III</td>
</tr>
<tr>
<td>Q99KA8 (Experimental evidence at transcript level)</td>
<td>No information</td>
<td>927</td>
<td>103 444</td>
<td>Missing 1–59 aa (ligand binding domain is not affected)</td>
<td>Ligand binding domain, fibronectin type III domain, juxtamembrane domain, protein kinase domain, SAM domain</td>
</tr>
<tr>
<td>A0A087WQZ6 (Protein predicted)</td>
<td>3043</td>
<td>38</td>
<td>4137</td>
<td>1–38 aa (missing the rest of domains)</td>
<td>No information</td>
</tr>
</tbody>
</table>

Note: The value “Experimental evidence at protein level” indicates that there is clear experimental evidence for the existence of the protein. The criteria include partial or complete Edman sequencing, clear identification by mass spectrometry, X-ray or NMR structure, good quality protein–protein interaction, or detection of the protein by antibodies. The value “Experimental evidence at transcript level” indicates that the existence of a protein has not been strictly proven but that expression data (such as existence of cDNA(s), RT-PCR, or Northern blots) indicate the existence of a transcript. The value “Protein predicted” is used for entries without evidence at protein, transcript, or homology levels.

*Taken from UniProt last modified 8 January 2015.
†Information obtained from Ensembl (http://asia.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG000000026235;r=1:77367185–77515088).
‡Information obtained from UniProt (http://www.uniprot.org/uniprot/Q03137#showFeatures).
166 bp), and EphA4^{rb-2J/rb-2J} mice with mutant profile (a single 149 bp band) (Fig. 4). All the RLFP analyses were subsequently confirmed by DNA sequencing. All mutant mice exhibited hopping gait features, whereas both wild-type and heterozygous mice were apparently normal.

**Genotypes obtained correlate with the EphA4 phenotype**

Among 63 samples that were sequenced, 18 were wild-type (Epha4^{+/+}), 35 were heterozygous (Epha4^{rb-2J/rb-2J}), and 10 were mutants (Epha4^{rb-2J/rb-2J}). The analysis showed that the deletion found on exon 9 of EphA4^{rb-2J/rb-2J} mice was 100% in concordance with the features observed in the mutant mice.

**Discussion**

The central pattern generators (CPGs) are the neuronal networks that generate and coordinate rhythmic limb movement. The hopping gait phenotype displayed by the EphA4 mutant is thought to be due to CPG neurons abnormally crossing the midline of the spinal cord (Kullander et al. 2003). The crossing generates the synchronous locomotion of the hind limbs through reciprocal over-excitation of the CPG neurons.

Previous studies aimed at understanding role of the functional domains of EphA4 receptor in the mouse revealed the requirement for kinase function in axon formation and guidance (Dufour et al. 2006; Egea et al. 2005; Kullander et al. 2001). The cytoplasmic domains of Eph receptor are the juxtamembrane (JM) domain, kinase domain, sterile-c-o-motif (SAM) domain, and PDZ domain. In functional studies, the hopping gait phenotype only appeared in EphA4 mutants (Epha4^{KD}) with a defective kinase domain (Kullander et al. 2001) and in mutants (Epha4^{GFP}) with the absence of the entire functional cytoplasmic domains (Egea et al. 2005). Another EphA4 mutant (Epha4^{GS}) displayed normal alternating gait when mutations of two tyrosine residues (Y596E and Y602E) were introduced in the JM domain (Egea et al. 2005). Despite the decrease of auto-phosphorylation in the mutant, there was an increase of basal kinase activity almost similar or higher than ephrin-activated EphA4. This would explain the requirement of kinase activity for normal functioning of CPG neurons and a normal alternation of gait. The findings support the hypothesis that tyrosine residues in the JM domain regulate EphA4 kinase activity. The mutation in EphA4^{rb-2J/rb-2J} is located at E582 prior to the major auto-phosphorylation sites. The deletion resulted in a frameshift, leading upon translation to altered six encoded codons followed by a stop codon. The EphA4^{rb-2J/rb-2J} protein is therefore truncated, lacking the tyrosine auto-phosphorylation sites in the JM domain, the kinase domain, SAM domain, and PDZ domain. The phenotype of the EphA4^{rb-2J/rb-2J} mouse differs from the other EphA4 mutants that have been published in the past in that it not only hops but also leans. Therefore, variability in phenotypic representations appears to result from differing mutations within the same gene (Table 2). Parallel analysis of the differing mouse lines using identical methodology would be required to further investigate this question.

Ephs and ephrins are known to exist as a number of splice variants in both humans (Finne et al. 2004) and mice (Holmberg et al. 2000). Seven isoforms of the EphA4 protein have been annotated (available from UniProt; Table 3). It seems likely that the EphA4^{rb-2J/rb-2J} protein exists in two variants, with the full-length protein being absent. First, truncation of the protein owing to the exon 9 mutation would result in a protein that lacks the kinase domain and is therefore not detected by Western blot. Truncation of EphA4 was consistent with the Western blot analysis, in which the full-length EphA4 protein (110 kDa) was absent in homozygote mutant mice. If expressed, this truncated protein would be predicted to have a mass of 63 kDa, which is similar in size to the 63 kDa short isoform of EphA4 (Fig. 2D).

The Western blot results suggest that a second isoform of EphA4 is present with a mass of 104 kDa, which is similar in size but different from the isoforms predicted from UniProt (http://www.uniprot.org/uniprot?query=EphA4&sort=score; Fig. 2C). This band is hypothesised to correspond to a previously unknown isoform, which has exon 9 spliced out but still possesses the epitope located in the SAM domain, which is detected by the Santa Cruz antibody.

**Conclusions**

We identified a single nucleotide deletion of adenine in exon 9 of EphA4 in the EphA4^{rb-2J/rb-2J} mouse mutant. This deletion results in a frameshift, which is predicted to cause premature truncation of the protein, and lack of key cytoplasmic domains. This data are important in validating experiments that utilise the EphA4^{rb-2J/rb-2J} mouse line to investigate the function of the EphA4 gene. Furthermore, this study underlines the potential existence of multiple isoforms of the EphA4 protein. Possible variability in phenotype could arise owing to the presence of differing proportions of various isoforms.

**Conflict of Interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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