Electroretinography as a Screening Method for Mutations Causing Retinal Dysfunction in Mice

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PURPOSE. To detect mice with hereditary retinal impairment, a high-throughput electroretinography (ERG) screening system was established.

METHOD. Mice from eight different strains without known retinal disorders (102, 129/SvJ, AKR, C57BL/6J, C57BL/6Jlco, CBA/Caj, and DBA/2NCrBBr) and one control strain with retinal degeneration (C3HeB/FeJ) were fixed on a specially constructed sled, ERG electrodes were placed on the cornea, and mice were moved into a Ganzfeld stimulator. From a luminance range of 0.0125 to 500 cd-s/m² in a pretest series two levels (5 and 125 cd-s/m²) were chosen to shorten examination times. The root mean square (RMS) of the ERG-recording was analyzed to detect animals with abnormal retinal function. ERG responses of the left and right eyes were compared in amplitudes and implicit times of the a- and b-waves. Statistical analysis of the latter parameters was performed in all wild-type animals. Histology was performed on selected mice.

RESULTS. ERG recordings of individual animals for the left and right eye revealed good agreement in amplitudes and implicit times of the a- and b-waves (P < 0.05). Comparison of these parameters among the wild-type strains showed several differences. Evaluation of the RMS revealed, in addition to the C3HeB/FeJ mice, a subgroup of mice within the 129/SvJ strain with abnormal retinal function. Molecular analysis of these mice demonstrated the presence of the same retroviral insertion in the Pde6b gene, which is causative of the Pde6b⁰ allele carried in C3HeB/FeJ mice. Histologic analysis demonstrated good correlation between retinal electrophysiology and morphology.

CONCLUSIONS. The present results demonstrate the feasibility of ERG for screening a large number of mice to detect animals with functional retinal impairment. (Invest Ophthalmol Vis Sci. 2004;45:601–609) DOI:10.1167/iovs.03-0561

Human retinal disorders cover a broad variety of clinical symptoms and many different genes are involved in the corresponding pathologic conditions. The two most important groups are retinitis pigmentosa (RP) and age-related-macular-degeneration (ARMD). In Germany, approximately 10% and 15% of legal blindness is caused by these two groups, respectively.¹ Worldwide, the prevalence of RP is approximately 1:4000. The reasons for the progressive loss of photoreceptors (PRs) are genetic abnormalities at more than 100 gene loci, affecting various ocular tissues (for a recent review, see Ref. 2; and corresponding web sites at http://eyegene.meei.harvard.edu or http://www.sph.uth.tmc.edu/RetNet). A few examples for these human disorders will be described.

ARMD is the leading cause of blindness in the elderly in developed countries. A complex series of events ultimately leads to degeneration of PRs. Various etiologies are currently proposed for this disease, with genetic factors considered as major components. ARMD is considered a genetically complex disease, but in contrast to RP, only candidate genes can be discussed.³ In contrast, there are some congenital diseases with a clear genetic background. One of them is Stargardt’s disease, which is transmitted mainly as an autosomal-recessive trait with a prevalence of 1:10,000. Mutations in a few genes have been shown to be causative of particular forms.⁴–⁶ Moreover, a combination of the clinical features of RP with deafness is referred to as Usher syndrome (USH); it is also transmitted as an autosomal-recessive trait with a frequency of 1:16,000 (for a recent review, see Ref. 7). In Usher syndrome, several subtypes have been reported, and causative mutations have been identified in a variety of genes (examples are given in Refs. 8–14).

Leber’s congenital amaurosis (LCA) affects children very early in life with a frequency of 1:40,000 in an autosomal-recessive or sometimes autosomal-dominant manner. It accounts for 10% to 18% of cases of congenital blindness. Several genes have been identified as involved in the pathogenesis of LCA, but mutations in RPE65 are responsible for 10% to 15% of all cases, and mutations in CRX are responsible for the dominant form of LCA (for a recent review, see Ref. 15). Later, teenagers and young adults are affected with a similar frequency (1:40,000) by cone–rod dystrophies (CORDs). Clinically, the CORDs are heterogeneous, which is reflected also by their genetic heterogeneity. CORD can be inherited in an autosomal-recessive, autosomal-dominant, or X-linked manner. Causative mutations have been identified in several genes.¹⁶–²³ Mutations in some genes are attributed to different disorders—for example, mutations in ABCA4, which lead to CORD3 and STGD1.¹⁴

Even if this short survey suggests that some retinal disorders can be attributed to mutations in particular genes, many
of these retinal diseases remain to be characterized functionally, to understand the various mechanisms underlying the clinical features of retinal degeneration and develop strategies for therapeutic intervention. Therefore, mouse models reflecting the broad variety of human genetic disorders would be desirable to characterize the physiological mechanisms in these phenotypic deviants. Most of the mouse models available to date were designed as transgenic or knockout mice. Several targeted mutations have been constructed that affect different ocular structures such as the retinal pigment epithelium (e.g., Rpe65, Rbp4, and Mertk) and transport and membrane channels (Cng3). However, most of the targeted mutations affect the photoreceptors (representative models affect Aber, arrestin, Crx, Rep1, Bho, Rom1, Rgr, or Tulp1). However, these null mutations do not reflect the clinical situation in all cases, because mutations in genes that affect the general metabolism (such as the RBP4 gene) also affect retinal function.

One reason is that many retinal disorders are transmitted as complex traits. Experimentally, the transmission of these disorders can be investigated by the use of double transgenics or double knockouts. One example (Rpe65<sup>−/−</sup>/Cnga3<sup>−/−</sup>) has been published recently. However, spontaneous or randomly induced mouse mutations may be closer to the human situation. Only a few such mouse mutations have been described, such as the rd1 mutation as a retroviral integration in the Pde6b gene, the rds/rd2 mutation affecting the Prph2 gene, or the or1 allele in the Chx10 gene. A list of an additional 16 spontaneous mouse mutants affecting retinal function was published recently, but only a few have been characterized at a molecular level and assigned to known genes such as Pde6b, Prph2, or Mitf.

Therefore, we established a systematic screen for mutations in mice using a functional assay. Herein, we report the analysis of the baselines in various wild-type strains of mice. We detected the Pde6b mutant allele segregating in strain 129/SvJ, which was considered previously to be wild-type with respect to retinal degeneration. This finding demonstrates the feasibility of the current method, which allows in the German Mouse Clinic (GMC; http://www.gsf.de/ieg/gmc/index.html) a high-throughput screening of mice derived from parents treated by the mutagenic agent ethylnitrosourea (ENU).

**Methods**

**Animals**

Eight different mouse strains, which were considered up to now in many laboratories to be wild-type for retinal degeneration, were included in the study: 102 (n = 8), 129/SvJ (n = 10), AKR (n = 7), C57BL/6J (n = 10), C57BL/6Jo-co (n = 10), CBA/CaJ (n = 11), DBA/2NCrBR (n = 6), and JF1 (n = 6). As an established control for hereditary retinal degeneration we used mice from the strain C3H/HeJ/Fel (n = 10). If not otherwise stated, mice were 6 weeks old at the time of analysis. DBA/2NCrBR mice were bought from Charles River (Kisslegg, Germany). All other strains were bred in the GSF Research Center. Experiments and housing of the animals were performed according to the German Law on the Protection of Animals (Regulation 209-2531-55/01 by the Government of Upper Bavaria) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Electroretinography**

Mice were dark adapted for at least 12 hours and anesthetized with 137 mg ketamine and 6.6 mg xylazine per kilogram body weight. After pupil dilation (1 drop atropine 1%), individual mice were fixed on its sled into the position for examination with both eyes within the Ganzfeld bowl.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933227/)

**Figure 1.** Electroretinography on fixed, anesthetized mice. The handheld Ganzfeld stimulator is fixed at the bottom, and the mouse is moved on its sled into the position for examination with both eyes within the Ganzfeld bowl.

**Figure 2.** Intensity series of corneal electroretinograms in one representative strain C57BL/6J mouse. (A) Traces show averages of 10 individual responses to a luminance of (1) 0.0125; (2) 0.025; (3) 0.125; (4) 0.5; (5) 1.25; (6) 5; (7) 12.5; (8) 50; (9) 125; and (10) 500 cd-s/m². For screening purposes in all other groups, 5 and 125 cd-s/m² were chosen as step 1 and step 2 (bold traces), respectively, to shorten examination times. (B) (●) amplitudes of the b-waves. The line shows the Naka-Rushton fit in this mouse.
measurements at each step. A bandpass filter was set to range from 0.15 to 1000 Hz.

TABLE 1. Bland-Altman Analysis of Amplitude and Implicit Time of Right and Left Eyes in Steps 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>a-Wave</th>
<th>b-Wave</th>
<th>Implicit Time (ms)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Step 1</td>
<td>Step 2</td>
<td>Step 1</td>
</tr>
<tr>
<td>Average of all eyes</td>
<td>-11.2</td>
<td>-35.1</td>
<td>140.5</td>
</tr>
<tr>
<td>Mean of right-left eye</td>
<td>1.4</td>
<td>1.6</td>
<td>2.9</td>
</tr>
<tr>
<td>SD of right-left eye</td>
<td>4.5</td>
<td>12.4</td>
<td>42.9</td>
</tr>
<tr>
<td>Percentage of eyes within 2 SD-range</td>
<td>95.1</td>
<td>95.2</td>
<td>95.8</td>
</tr>
</tbody>
</table>

Statistics
A specially written computer program (MatLab; The MathWorks, Natick, MA) was used to search for minima and maxima to determine a- and b-waves automatically (a-wave amplitude was defined as the minimum between 0 and 50 ms; b-wave amplitude was defined as the difference between the a-wave and the maximum between 30 and 200 ms). The Naka-Rushton fits for the amplitude of the b-wave were iteratively performed. Data retrieved by the computer program were checked individually by an experienced electrophysiologist to ensure that the proper peaks have been detected. Special attention had to be paid to the correct determination of the b-wave in step 2 to exclude bias from overlying oscillatory potentials (OPs), although in most cases, none of the large OPs caused interference. The following comparisons were performed: (1) root mean square (RMS) of ERG recordings (between 0 and 200 ms) of individual mice to detect animals with functional impairment; (2) amplitude and implicit time of a- and b-waves in step 1 and 2 in all mice, to assess the agreement between left and right eyes; and (3) amplitude and implicit time of a- and b-waves in all mice without known retinal impairment, to assess differences between groups considered normal. Because measurements were made across eight different strains (which were initially thought to be wild-type with respect to retinal degeneration) and one blind control strain carrying the Pde6brd1 mutation with the possibility of multiple pair-wise comparisons, the significance of the differences between (1) and (3) were evaluated by ANOVA followed by the Scheffe post hoc F test. In (2) a Bland-Altman analysis was performed to reveal any relationship between the differences in the right and left eyes and their averages.

Morphologic Analysis
For the morphologic analysis and documentation of retinal degeneration, eyes were fixed for 24 hours in Davidson solution, dehydrated, and embedded in paraffin or plastic medium (JB4-Plus; Polysciences, Inc., Eppelheim, Germany) according to the manufacturer’s procedure. Sectioning was performed with an ultramicrotome (Ultratom OMU3; Reichert, Walldorf, Germany). Serial transverse 2-μm sections were cut with a dry glass knife and collected in water drops on glass slides. After drying, the sections were stained...
with methylene blue and basic fuchsin and evaluated with a light microscope (Axioplan; Carl Zeiss Meditec, Göttingen, Germany). Images were acquired by means of a scanning camera (Axiocam; Carl Zeiss Meditec) equipped with a screen-capture program (KS100; Carl Zeiss Vision, Hallbergmoos, Germany) and imported into an image-processing program (Illustrator, ver. 9.0, or Photoshop, ver. 6.0; Adobe, Unterschleissheim, Germany).

Isolation of DNA and PCR Conditions

Genomic DNA was prepared from tail tips of the mice according to standard procedures. To detect the Xmr-28 insertion in the Pde6b gene leading to retinal degeneration (rd1), we amplified the corresponding region using the primers 10 and JS610 essentially as described previously47 with 35 cycles (MJ Research PTC-225 machine; Biozym Diagnostik GmbH, Hess, Oldendorf, Germany) using 67°C annealing temperature. PCR products were separated in a 1% agarose gel.

General

Chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany). The enzymes and ingredients for PCR were from Invitrogen or Roth (both Karlsruhe, Germany) or from MBI Fermentas (St. Leon-Rot, Germany).

RESULTS

From a preliminary series with 10 C57BL/6J mice at 10 different luminance levels two levels at 5 and 125 cd-s/m² were chosen that produced reliable responses and easily detectable peaks and represented two quite different illumination ranges, and because a luminance of 5 cd-s/m² (step 1) evoked a well discernible b-wave amplitude mainly stemming from the rod system, whereas 125 cd-s/m² (step 2) evoked a saturated b-wave response with an a-wave, mediated presumably by a mixed rod and cone input (Fig. 2A). The restriction to a two-step screening system shortened the examination time and provided stable measurement conditions. The setup used in this study allowed two people to screen 20 to 25 mice every 4 hours.

One subset of five animals of the 129/SvJ strain showed no recognizable a- or b-waves and C3HeB/FeJ mice showed greatly diminished ERG responses (step 1: one animal with recognizable, but diminished a- and b-waves; step 2: five animals with recognizable, but diminished a- and b-waves). These two groups were found to be significantly different in terms of the RMS in all other groups in step 1, including the remaining normal mice of strain 129/SvJ. In step 2, all groups except JF1 were significantly different from the subset of 129/SvJ and C3HeB/FeJ mice (Fig. 2A). Retinal impairment in these two groups (129/SvJ and C3HeB/FeJ) were confirmed by morphologic examination (described later) and excluded from further analysis of ERG components. In addition, we found statistically significant differences of the RMS between the following normal strains: JF1 versus 102, JF1 versus AKR, JF1 versus CBA/CaJ, CBA/CaJ versus C57BL/6J, and CBA/CaJ versus DBA/2N (Fig. 3).
mice, which are homozygous in our C57BL/6J colony six age points by ERG (at 125 cd-s/m²). All other strains tested were negative for this insertion (Fig. 6).

Pde6brd1 animals were big enough to be easily age. At this age, the mice had a fully developed retina, and the measurement. However, we chose 6 weeks of age in accordance with previous studies.49-50 Even if it is a compromise to minimize animal handling and housing costs by screening animals as early as possible, we were able to detect all animals with retinal degeneration correctly when examined at 6 weeks of age.

### Table 2. ERG Parameters of Each Normal Group

<table>
<thead>
<tr>
<th>Group</th>
<th>a-Wave</th>
<th>b-Wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step 1</td>
<td>Step 2</td>
</tr>
<tr>
<td>Amplitude [μV]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>−12.2 ± 3.3</td>
<td>−27.7 ± 12.6</td>
</tr>
<tr>
<td>129/SvJ</td>
<td>−12.6 ± 4.7</td>
<td>−22.5 ± 10.0</td>
</tr>
<tr>
<td>AKR</td>
<td>−11.1 ± 2.1</td>
<td>−38.5 ± 15.0</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>−10.5 ± 3.4</td>
<td>−41.6 ± 8.6</td>
</tr>
<tr>
<td>C57BL/6jco</td>
<td>−7.9 ± 4.5</td>
<td>−39.8 ± 17.5</td>
</tr>
<tr>
<td>CBA/Caj</td>
<td>−12.9 ± 5.3</td>
<td>−20.7 ± 11.6</td>
</tr>
<tr>
<td>DBA/2NCrBR</td>
<td>−10.9 ± 7.3</td>
<td>−32.4 ± 7.1</td>
</tr>
<tr>
<td>SJ3</td>
<td>−13.5 ± 7.2</td>
<td>−36.1 ± 7.5</td>
</tr>
<tr>
<td>Implicit time [ms]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>23.4 ± 5.6</td>
<td>26.4 ± 3.0</td>
</tr>
<tr>
<td>129/SvJ</td>
<td>23.5 ± 7.5</td>
<td>26.4 ± 3.6</td>
</tr>
<tr>
<td>AKR</td>
<td>25.7 ± 2.1</td>
<td>25.7 ± 1.7</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>28.3 ± 9.2</td>
<td>25.9 ± 2.2</td>
</tr>
<tr>
<td>C57BL/6jco</td>
<td>29.6 ± 5.9</td>
<td>28.6 ± 5.5</td>
</tr>
<tr>
<td>CBA/Caj</td>
<td>24.0 ± 3.5</td>
<td>22.1 ± 3.9</td>
</tr>
<tr>
<td>DBA/2NCrBR</td>
<td>30.5 ± 4.3</td>
<td>29.8 ± 2.0</td>
</tr>
<tr>
<td>SJ3</td>
<td>36.2 ± 4.0</td>
<td>29.6 ± 3.5</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SD. Each value shows the average of the right and left eye. No statistically significant differences were found except for the following: b-Wave amplitude step 1: 129/SvJ and AKR, AKR and DBA/2NCrBR, and AKR and JF1; all at P < 0.05. a-Wave implicit time step 1: CBA/Caj and DBA/2NCrBR, CBA/Caj and JF1; all at P < 0.05. b-Wave implicit time step 1: 102 and AKR, AKR and CBA/Caj; all at P < 0.05. b-Wave implicit time step 2: CBA/Caj and JF1 at P < 0.05; 102 and JF1 at P < 0.01.

Subsequently, Bland-Altman analysis of the amplitudes and implicit times of a- and b-waves of steps 1 and 2 in the right and left eyes (Table 1) did not reveal any systematic or proportional error or any dependence on the magnitude of the values. According to the suggestion of Bland and Altman,46 the two measurements can be used interchangeably or, in our case, the left and right eyes can be averaged for further detailed statistical evaluation (Fig. 4). Within the eight strains of normal mice, a- and b-waves were compared in steps 1 and 2 for amplitude and implicit time. The Scheffé post hoc F test revealed three statistically significant differences in amplitude and six differences in implicit time (Table 2).

Histologic evaluation of all mice with a typical wild-type ERG response showed well-ordered retinal layers. C3HeB/FcJ mice, which are homozygous Pde6brd1, showed no ERG response and had degenerated photoreceptor layers. The overall histologic appearance of the retinas of the 129/SvJ mice was similar to that of C3HeB/FcJ (Fig. 5).

Because the retinal degeneration in the C3HeB/FcJ mouse strain had been demonstrated to be caused by a retroviral insertion into the Pde6b gene, we tested for the presence of the Pde6b<sup>att</sup> allele in all strains used during this study. The presence of the Pde6b<sup>att</sup> allele in our C3HeB/FcJ mice was confirmed. All 129/SvJ mice were found to be homozygous for the Pde6b<sup>att</sup> allele. Among those 129/SvJ mice with a normal ERG response, we found some to be carrying the Pde6b<sup>dfl</sup> allele, presumably heterozygous (data not shown). All other strains tested were negative for this insertion (Fig. 6).

To identify the most feasible age of screening, we checked in our C57BL/6J colony six age points by ERG (at 125 cd·s/m²). The ERG response increased and reached its maximum at 8.5 weeks of age (Fig. 7), which is in good agreement with Li et al.,48 who have shown that saturation is reached at 8 weeks of age. At this age, the mice had a fully developed retina, and the animals were big enough to be easily fixed on the sled for ERG measurement. However, we chose 6 weeks of age in accordance with previous studies.49-50 Even if it is a compromise to minimize animal handling and housing costs by screening animals as early as possible, we were able to detect all animals with retinal degeneration correctly when examined at 6 weeks of age.

### Discussion

We successfully developed a high-throughput protocol on the basis of ERG as a quick, robust, and reproducible in vivo screening method for inherited functional retinal impairment in mice. The data obtained demonstrate that there is no major left-right difference in the ERG responses. However, in each strain, individual mice showed variation, making further statistical evaluation necessary in most cases. Two groups, one of them being C3HeB/FcJ and one of them consisting of a subset of five animals of the 129/SvJ strain, showed either no discernible a- or b-wave (10 animals) or greatly diminished amplitudes. The animals with no discernible a- or b-wave are easily detected during the screening process and showed significantly lower RMS values in the ERG recordings.

For screening purposes the b-wave amplitude seems to be the most stable and informative end point, because of its magnitude and high sensitivity due to the input of the originating cells. We suggest that outliers be defined as those animals with ERG responses outside the 95% confidence interval (which was calculated from the normal subjects in the present study). Animals with ERG responses less than the lower limit should undergo further ERG evaluation before undergoing the histologic and genetic tests. The lower limits calculate to 76 μV (step 1) or 97 μV (step 2) for amplitude. All animals with morphologically and genetically certain retinal degeneration (as proven later in the process by histology and genetical analysis) were correctly classified based on our defined criterion with respect to amplitude of the b-wave. This
criterion is easily checked by the technician during a fast screening process, to detect animals with a suspected retinal disease to take appropriate action in the later evaluation process.

The method suggested above was validated by the examination of C3HeB/FeJ mice, which are known to be homozygous for the mutant allele \textit{Pde6brd1}.\textsuperscript{38} They showed ERG responses significantly reduced in comparison to all wild-type strains.

\textbf{FIGURE 5.} The ERG response corresponded to retinal histology. The ERG response of one mouse is given together with the histology of its retina. The mouse strains used are C57BL/6J, C57BL/6Jico, C3HeB/FeJ, 129/SvJ\textsuperscript{blind}, 129/SvJ, CBA/CaJ, DBA/2NCrI\textsuperscript{BR}, 102, and AKR. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; PE, pigmented epithelium; OPL, outer fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer.
strains. Moreover, the screening method is also robust for the detection of individual mice with impaired retinal function as demonstrated for a subset of mice from the 129/SvJ strain. In these mice, the ERG response showed no discernible a- or b-waves. They were found to be statistically different in their RMS value. As a first molecular characterization, we checked all our strains investigated for the presence or absence of the retroviral insertion causative of the \( \text{Pde6b}^{rd1} \) allele.\cite{58} Those 129/SvJ mice with no discernible ERG response were shown to be homozygous \( \text{Pde6b}^{rd1} \). Among the 129/SvJ with normal ERG, some were homozygous wild type and others were heterozygous \( \text{Pde6b}^{rd1} \), indicating that the \( \text{Pde6b}^{rd1} \) allele segregates in our 129/SvJ stock and is expressed recessively. Moreover, these results validate the method applied to identify outliers such as 129/SvJblind.

The strain 129 is widely used for the generation of targeted mutations (knockouts). A large degree of genetic diversity among the 129 substrains was recently identified.\cite{51,52} In particular, the strain 129/SvJ was genetically contaminated in approximately 1978 by an unknown strain and differs from other 129 substrains at approximately 25% of simple sequence length polymorphism (SSLP) genetic markers. Therefore, it is most likely that it affects also the presence of the \( \text{Pde6b}^{rd1} \) allele in other 129/SvJ colonies.

In conclusion, the newly established system reported herein is quick and sensitive and allows a high-throughput screening in mice. Therefore, we will apply this system to screen ENU-mutagenized mice to develop new mouse models of retinal degeneration.

**FIGURE 6.** Molecular analysis for the presence of the \( \text{Pde6b}^{rd1} \) allele. Genomic DNA of all mouse strains tested in this study was checked by PCR for the presence or absence of the \( \text{Pde6b}^{rd1} \) allele. C3HeB/FeJ mice as well as in 129/SvJblind mice with lowered ERG response were shown to be homozygous \( \text{Pde6b}^{rd1} \) carriers. Some presumably heterozygous carriers were identified among the 129/SvJ mice with normal ERG. The empty PCR control is indicated by a slash; arrow: expected size at 3.3 kb.

**FIGURE 7.** Age-dependence of retinal development and ERG response. Histologic sections and the corresponding ERG is given for the age points 3.5, 4, 6, 8, 8.5, and 11 weeks.
Acknowledgments

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References


