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Research Report

Modified sound-evoked brainstem potentials in *Foxp2* mutant mice

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ABSTRACT

Heterozygous mutations of the human *FOXP2* gene cause a developmental disorder involving impaired learning and production of fluent spoken language. Previous investigations of its aetiology have focused on disturbed function of neural circuits involved in motor control. However, *Foxp2* expression has been found in the cochlea and auditory brain centers and deficits in auditory processing could contribute to difficulties in speech learning and production. Here, we recorded auditory brainstem responses (ABR) to assess two heterozygous mouse models carrying distinct *Foxp2* point mutations matching those found in humans with *FOXP2*-related speech/language impairment. Mice which carry a *Foxp2*-S321X nonsense mutation, yielding reduced dosage of *Foxp2* protein, did not show systematic ABR differences from wildtype littermates. Given that speech/language disorders are observed in heterozygous humans with similar nonsense mutations (*FOXP2*-R328X), our findings suggest that auditory processing deficits up to the midbrain level are not causative for *FOXP2*-related language impairments. Interestingly, however, mice harboring a *Foxp2*-R552H missense mutation displayed systematic alterations in ABR waves with longer latencies (significant for waves I, III, IV) and smaller amplitudes (significant for waves I, IV) suggesting that either the synchrony of synaptic transmission in the cochlea and in auditory brainstem centers is affected, or fewer auditory nerve fibers and fewer neurons in auditory brainstem centers are activated compared to wildtypes. Therefore, the R552H mutation uncovers possible roles for *Foxp2* in the development and/or function of the auditory system. Since ABR audiometry is easily accessible in humans, our data call for systematic testing of auditory functions in humans with *FOXP2* mutations.

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1. Introduction

Heterozygous mutations of the *FOXP2* gene cause the best described example of an inherited speech and language disorder in humans (Lai et al., 2001; MacDermot et al., 2005).

It is characterized by impaired learning/production of complex oral movements underlying speech, accompanied by linguistic deficits which affect both spoken and written modalities (Watkins et al., 2002; see Fisher, 2006 for review). *FOXP2* encodes a forkhead-box transcription factor with a

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Abbreviations: ABR, auditory brainstem response; ENU, N-ethyl-N-nitrosourea; *Foxp2*, forkhead-box p2; SPL, sound pressure level; WT, wildtype

characteristic DNA-binding domain, and acts to regulate expression of many downstream target genes (Vernes et al., 2007). The gene is found in highly similar form in many vertebrate species, including rodents, where it is expressed in corresponding neuronal subpopulations of the brain (Fisher and Scharff, 2009). Recently, an ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis strategy enabled the generation of mouse models carrying distinct *Foxp2* point mutations (single nucleotide changes) that match those observed in humans with speech/language deficits (Groszer et al., 2008).

Foxp2-R552H mice recapitulate the human *FOXP2*-R553H missense mutation found in a particularly well-studied three-generation family, known as the KE family; this yields an amino-acid substitution in the encoded protein, replacing an arginine residue with a histidine at one crucial site within the DNA-binding domain (Lai et al., 2001). In cell-based studies, the mutated protein shows abnormal intracellular localization, impaired DNA-binding and disturbed regulation of transcriptional activity of targets (Vernes et al., 2006). *Foxp2*-S321X mice carry a different type of point mutation, known as a nonsense mutation, which creates a premature stop codon midway through the gene, and is thus expected to encode a truncated protein lacking the DNA-binding domain and other key functional regions. In fact, *in vivo* analyses indicate that this allele does not produce *Foxp2* protein at all, most likely due to a combination of nonsense-mediated RNA decay (degradation of mRNA transcripts carrying the nonsense mutation) and instability of any truncated protein that does get produced (Groszer et al., 2008; Vernes et al., 2006). The *Foxp2*-S321X mutation is very similar to a human *FOXP2*-R328X nonsense mutation found in all three affected members of another family segregating speech and language deficits (MacDermot et al. 2005).

Mice that are homozygous for the above mutations display severe reductions in cerebellar growth and postnatal weight gain, as well as profound general motor impairment, and die after 3–4 weeks. Heterozygous mice are fully viable and gain weight normally, but show deficits in motor-skill learning and synaptic plasticity, including a lack of long-term depression in the dorsolateral striatum (Groszer et al., 2008). Both heterozygous and homozygous pups produce innate ultrasonic vocalizations despite *Foxp2* disruption (Groszer et al., 2008).

Speech learning and production make heavy demands on rapid and fine motor control, but also depend on sensory processing within the auditory system (Fitch et al., 1997; Smith and Spirou, 2002). Thus, deficits in such sensory pathways could potentially contribute to impaired speech and language development (Hill et al., 2005). People carrying *FOXP2* mutations have been reported to have overtly normal hearing (Hurst et al., 1990). However, to our knowledge, no formal quantitative assessments of auditory abilities have yet been carried out, either in humans with *FOXP2*-related speech/language disorder, or in *Foxp2* mutant mice. Therefore, we used auditory brainstem response (ABR) audiometry which employs scalp electrodes to record sound-evoked bioelectrical potentials and to assess the peripheral auditory function of heterozygous *Foxp2* mutant mice in comparison to wildtype (WT) littermates. The ABR method has widely been applied to identify hearing deficits related to the auditory pathway from the cochlea up to the auditory midbrain in mice of various

genetic backgrounds (e.g. Shvarev, 1994; Willott et al., 1995; Trune et al., 1996; Reimer et al., 1996; Zheng et al., 1999; Burkard et al., 2001). Five peaks in the ABR waves are expected. In the mouse, peak I is suggested to refer to cochlear processing, peak II to processing in the cochlear nucleus complex, peak III in the complex of the superior olive, peak IV in the lateral lemniscus and peak V in the colliculus inferior.

In this context, physiological investigations of mouse models with *Foxp2* point mutations matching those that cause human speech/language impairment provide a valuable opportunity to identify novel aspects of aetiology in this disorder, and shed new light on functions of the gene. Furthermore since ABR measurements can easily be employed in humans, this method is useful for model validation and cross species comparisons.

2. Results

The analyses of hearing sensitivity (ABR thresholds), and amplitudes and latencies of the five peaks of the ABR waves identified differences in sound processing in the auditory periphery and brainstem of the *Foxp2* mutant mice. In Fig. 1 examples of the mean ABR of R552H and S321X heterozygous mutants and corresponding WT littermates are shown in response to 16 kHz tone bursts at a sound pressure level set to 20 dB above threshold. Five response peaks (I–V) with different latencies could be identified for all mice tested. No obvious

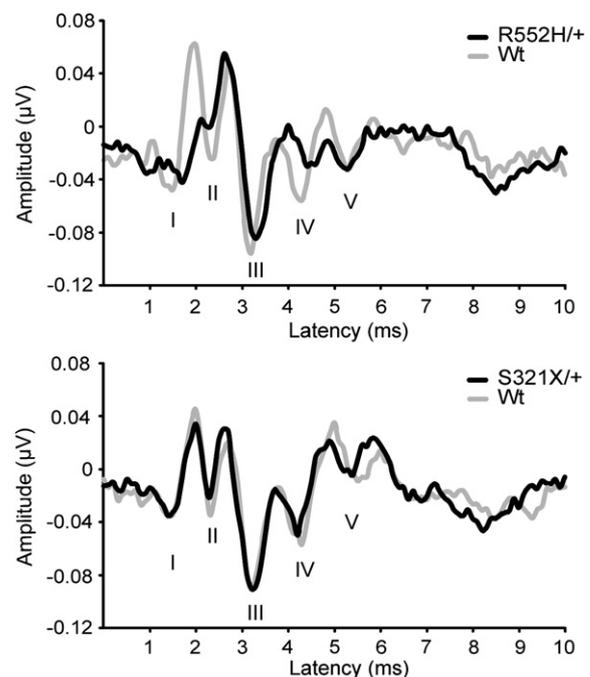


Fig. 1 – Examples of mean ABR recordings of R552H and S321X heterozygotes in comparison with their WT littermates. All recordings shown were obtained with a standard stimulus of 16 kHz tone bursts at 20 dB above response threshold. Recordings start with sound arrival at the ear (0 ms). Roman numerals indicate peaks I to V of the ABR waves.

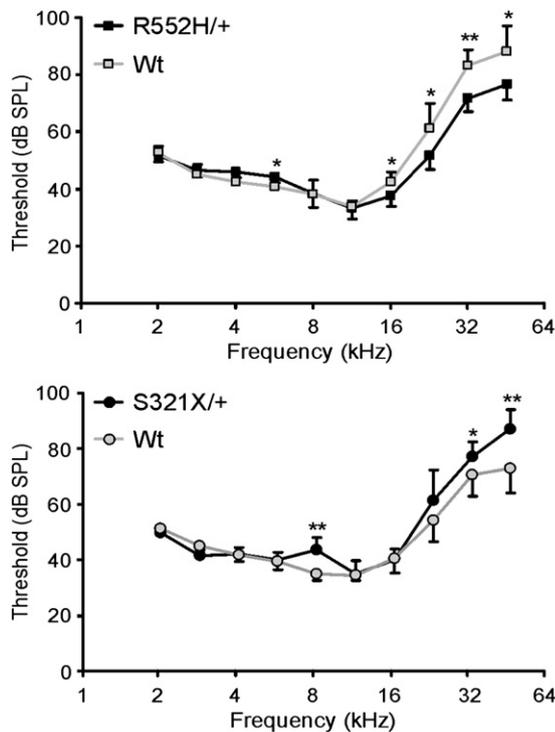


Fig. 2 – Mean ABR response thresholds. Data with standard deviations (shown only unilaterally for clearness) from heterozygotes and their WT littermates are plotted as a function of the tone frequency. Statistical significance by means of Student’s two-tailed t-test is indicated by asterisk (* $P < 0.05$, ** $P < 0.01$).

differences were seen in the wave patterns between S321X heterozygotes and WT littermates (Fig. 1, bottom). There was, however, a difference visible between the wave pattern in R552H heterozygotes compared to their WT littermates (Fig. 1, top). Peaks I and IV had smaller amplitudes and longer latencies in the mutants.

Fig. 2 illustrates ABR thresholds for all four groups of animals. The thresholds measured in the R552H heterozygotes were significantly lower in the high-frequency range (16–45.25 kHz; $P < 0.05$ or $P < 0.01$) indicating increased sensitivity compared to those of their WT littermates. There was also a

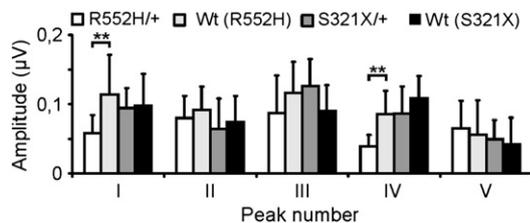


Fig. 3 – Mean amplitudes of ABR peaks I to V for all animal groups. Each peak amplitude corresponds to the average value of measurements obtained with a stimulus frequency of 16 kHz and sound pressure level 20 dB above threshold. Error bars show standard deviations. Statistical significance by means of Student’s two-tailed t-test is indicated by asterisk ($P < 0.01$).**

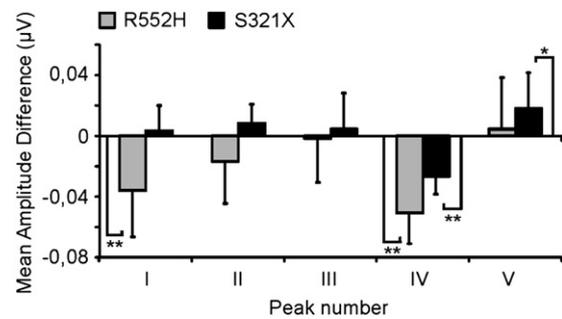


Fig. 4 – Mean difference (heterozygote minus wildtype) of ABR peak amplitudes averaged across all stimulation frequencies. Statistical significance by means of one sample t-test is indicated by asterisk (* $P < 0.05$, ** $P < 0.01$).

small but significant ($P < 0.05$) opposite difference at 5.66 kHz. In contrast, S321X heterozygotes had significantly higher thresholds indicating reduced sensitivity compared to their WT littermates at 8, 32, and 45.25 kHz ($P < 0.05$ or $P < 0.01$).

Closer examination of the peak amplitudes (Fig. 3) at 16 kHz tone bursts, the frequency range in which house mice are most sensitive (Ehret, 1979; Reimer et al., 1996), and a sound pressure level of 20 dB above threshold revealed statistically significant differences for peaks I and IV in the R552H group. Heterozygotes showed significantly smaller peak-to-trough amplitudes compared to WT animals for peaks I and IV ($P < 0.01$). In the S321X group, peak amplitudes at 16 kHz did not differ significantly for any peak between WT animals and heterozygotes.

In order to include all the test frequencies in a comparison between heterozygotes and WT animals, we calculated the average ABR amplitude at each test frequency separately for each peak and for all four experimental groups of animals. Then we calculated the difference between these average peak amplitudes from the heterozygotes and their respective WT littermates (heterozygote minus WT) and averaged these differences over all test frequencies separately for each peak. The results as shown in Fig. 4 indicate whether there are differences between heterozygotes and their WT littermates in the processing of tones of any frequency in the auditory brainstem centers related to the peaks of the ABR waves. A significantly smaller amplitude ($P < 0.01$) is obvious for peaks I

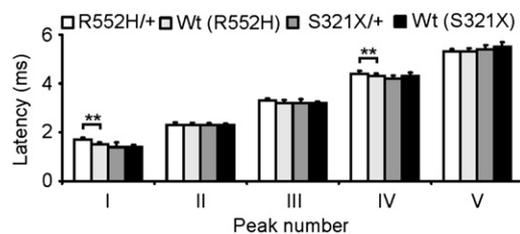


Fig. 5 – Mean latencies and standard deviations of ABR peaks I to V for all animal groups. Each peak latency corresponds to the average value of measurements obtained with a stimulus frequency of 16 kHz and sound pressure level 20 dB above threshold. Statistical significance by means of Student’s two-tailed t-test is indicated by asterisk ($P < 0.01$).**

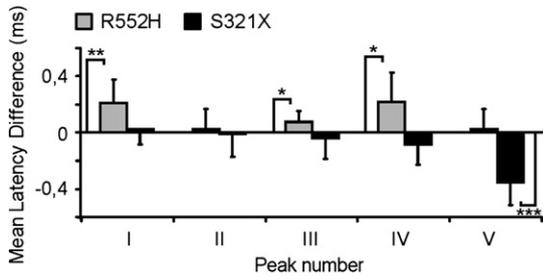


Fig. 6 – Mean difference (heterozygote minus wildtype) of ABR peak latencies averaged across all stimulation frequencies. Statistical significance by means of one sample t-test is indicated by asterisk (* $P < 0.05$, ** $P < 0.01$, * $P < 0.001$).**

and IV of the R552H heterozygotes (the average differences are negative). In addition, the amplitudes of peaks IV and V differed between S321X heterozygotes and WT littermates ($P < 0.05$ or $P < 0.01$).

Besides these changes in ABR peak amplitude we also found statistically significant differences in the latencies of the peaks. Fig. 5 shows latency data relative to stimulus onset obtained after stimulation with tone bursts of 16 kHz at 20 dB above response threshold. R552H heterozygotes had significantly longer latencies ($P < 0.01$) compared to their WT littermates for peaks I and IV. In contrast, no significant differences were found between the S321X heterozygotes and WT animals.

Similar to Fig. 4, Fig. 6 shows for each peak the difference in peak latencies (heterozygotes minus WT) as averaged across all tested frequencies. A significantly longer latency is obvious

for peak I ($P < 0.01$), and peaks III and IV ($P < 0.05$) of the R552H heterozygotes (the average differences are positive). In addition, the latency of peak V is shorter ($P < 0.001$) in S321X heterozygotes as compared to WT littermates.

Fig. 7 shows mean latencies for peaks I–V as a function of the tone frequency. With increasing frequency the latencies of all peaks decreased in very similar ways, as would be expected due to the delay of activation of cochlear hair cells as introduced by the traveling time of the traveling wave along basilar membrane. With increasing number of the peaks, the averaged latencies increased by about 1 ms which equals the synaptic delays between the respective levels of the ascending auditory pathway. There were no systematic or significant differences in these patterns between the groups of animals.

3. Discussion

A number of studies have demonstrated the general usefulness of ABR measurements in mice for investigating the genetic bases of hearing impairments (e.g. Hunter and Willott, 1987; Shvarev, 1994; Willott et al., 1995; Trune et al., 1996; Reimer et al., 1996; Zheng et al., 1999; Zhou et al., 2006). In the present report we assessed whether there were alterations in auditory processing in *Foxp2* mouse mutants by means of ABR measurements. Based on findings from people carrying heterozygous *FOXP2* mutations it has been hypothesized that reduced dosage of functional *FOXP2* protein in humans leads to impaired speech and language development. To date, two aetiological point mutations of human *FOXP2* have been reported; the *FOXP2*-R553H missense mutation found in the

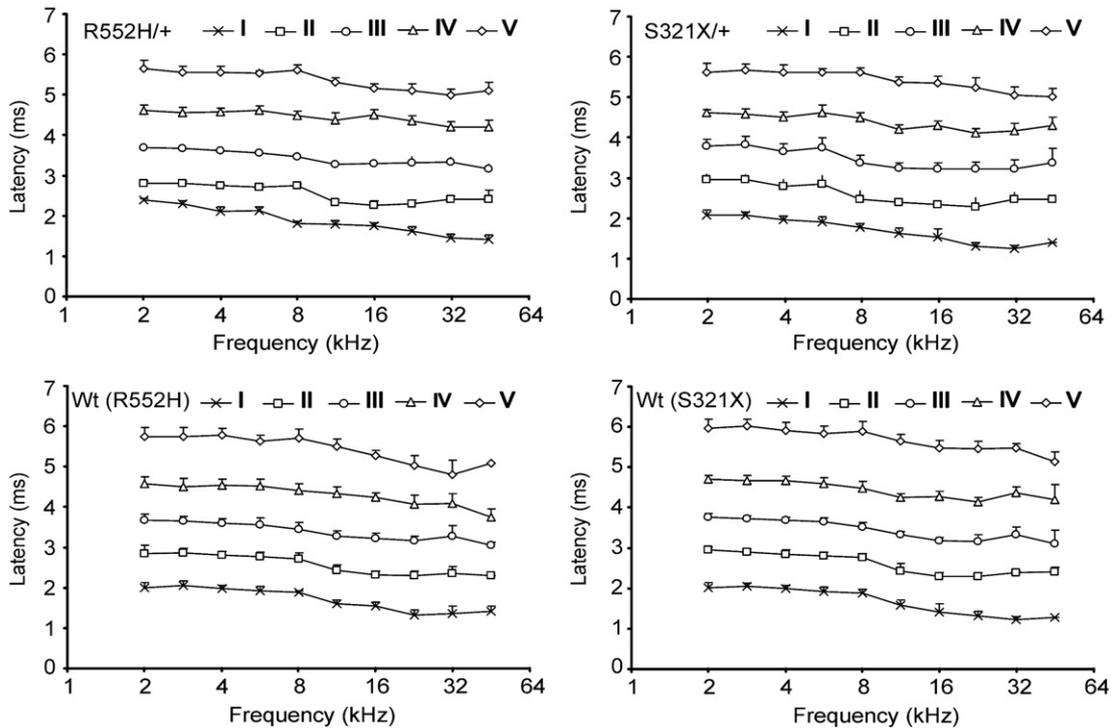


Fig. 7 – Comparison of mean latencies of ABR peaks I–V. Data with standard deviations are plotted as a function of tone frequency at tone levels of 20 dB above threshold. In all groups, latencies from WT and heterozygous animals decrease with rising tone frequency.

fifteen affected members of the large well-studied KE family (Lai et al., 2001), and the FOXP2-R328X nonsense mutation observed in all three affected members of a second smaller family (MacDermot et al. 2005). Notably, the mouse lines investigated in our study recapitulate these distinct mutations; *Foxp2*-R552H yields an identical amino-acid substitution to FOXP2-R553H, while *Foxp2*-S321X is a truncating mutation very close to FOXP2-R328X.

Both mutants were examined on the same genetic background, the C3H/HeN substrain. C3H strains have been demonstrated to have no hearing deficits up to at least 14 months of age (Ehret, 1979; Trune et al., 1996; Zheng et al., 1999). By using WT littermates as controls for our heterozygous mutants, any change in auditory function based on the genetic background should have been equally expressed in heterozygotes and WT controls. Thus, the observed differences in ABR characteristics between WT and heterozygous animals are likely to be based on the R552H or S321X mutation, respectively.

In general, ABR thresholds from previous measurements in mice were comparable to the data presented in our study (Zheng et al., 1999; Parham et al., 2001). Trune et al. (1996) reported similar thresholds for frequencies of 16 kHz and above in two substrains of C3H mice. Only for frequencies of 8 kHz and below they found elevated thresholds compared to those measured in the present study, which may be due to the different substrain of the mice used here.

Our study did not find evidence of gross hearing impairment in either *Foxp2*-S321X or *Foxp2*-R552H heterozygotes, but did uncover a number of significant alterations in auditory processing, with some intriguing differences between the distinct mutant lines. In the R552H heterozygotes, high-frequency hearing (16 kHz and higher, Fig. 2) was up to 12 dB better compared to WT animals. The high-frequency sensitivity (above the frequencies of best hearing) in mammals is determined mainly by the moment of inertia and frictional losses of the osseous chain of the middle ear and by the anatomy of the cochlea near its base (e.g. Dallos, 1973; Eldredge, 1974; Henson, 1974). One potential explanation of these data might be improved middle ear transmission for high frequencies due to smaller and stiffer middle ear ossicles, and/or a more efficient energy transfer to the cochlear hair cells in the high-frequency range due to anatomical differences in the cochlea. However, a contrasting pattern of results was seen for the S321X heterozygotes, which displayed decreased sensitivity in the high-frequency range, perhaps reflecting a reduced middle ear transmission for high frequencies and/or a less efficient energy transfer to the cochlear hair cells in the high-frequency range.

Although the ear is not noted as one of the major sites of *Foxp2* expression, in-situ hybridization studies of mouse embryos at embryonic day 13.5 have detected *Foxp2* mRNA in single cells of the developing inner ear (Gray et al., 2004; Mouse Genome Informatics Accession ID: 3507431). FOXP2 expression has also been documented in subtracted cDNA libraries prepared from developing human cochlea at 16–22 weeks of gestation (NCBI dbEST ID: 12768; Luijendijk et al., 2003). Our data on contrasting changes of high-frequency auditory sensitivity in the different *Foxp2* mouse lines calls for further studies of these mutants, aiming to find the anatomical and/or physiological bases of the observed changes.

Beyond the sensitivity differences noted above, S321X heterozygotes did not show any systematic modifications in ABR parameters as compared to control littermates. In contrast, we observed multiple differences for R552H heterozygotes not only for thresholds, but also for wave amplitudes and latencies. In particular, the R552H heterozygotes had a tendency across all tested frequencies to smaller peak amplitudes (significant for peaks I and IV, Fig. 4) and longer peak latencies (significant for peaks I, III, IV, Fig. 6) compared to their WT littermates. Since peaks of ABR waves represent the sum of synchronously occurring postsynaptic potentials at centers of the auditory pathway (Maurer et al., 1982; Parham et al., 2001), these deviations suggest that the R552H heterozygotes might have impaired synchronization of synaptic transmission along auditory pathways starting with the synapses between the cochlear hair cells and the auditory nerve fibers. A slight desynchronization in the information transfer through many channels automatically leads to smaller amplitudes and longer latencies of the summed postsynaptic potentials. The mechanism responsible for the desynchronization has to be clarified in further tests.

Our distinct phenotypic findings for S321X and R552H lines could relate to the differing nature of the mutations. Previous studies have shown that the S321X nonsense mutation yields a lack of *Foxp2* protein and is effectively a null allele (Groszer et al., 2008). S321X heterozygotes show a simple reduced dosage of functional *Foxp2* protein (around half the levels of wildtypes); this is also likely to apply to human heterozygotes carrying the R328X mutation (MacDermot et al., 2005). In contrast, rather than yielding absence of *Foxp2* protein product, the mouse R552H missense mutation (and its human equivalent FOXP2-R553H) leads to stable expression of a full-length mutant protein carrying an amino-acid substitution in its DNA-binding domain, one which disrupts its function as a transcription factor (Vernes et al., 2006). Therefore, although R552H heterozygotes do indeed show a reduced dosage of functional *Foxp2* protein, there could also be further effects mediated by the presence of the dysfunctional R552H form of the protein. For example, *Foxp2* proteins are known to act as dimers (two molecules bound together), and it is possible that mutant R552H protein might bind to wildtype protein, to have consequences beyond a straightforward loss of function (Vernes et al., 2006). Despite these potential mechanistic differences, it should be stressed that in humans the R328X and R553H mutations each lead to speech and language disorder, regardless of mutation type. We did not find any consistent alterations in auditory processing that were common to the heterozygous S321X and R552H mouse mutants. Thus, our findings would argue against the idea that FOXP2-related speech and language disorder is purely secondary to auditory processing deficits, and they require a more complex interpretation.

Overall, these results illustrate the value of studying an allelic series of mouse models carrying distinct aetiological mutations in the same gene. We observed *in vivo* differences in the impacts of distinct *Foxp2* mutations on ABR measures, which are interesting in light of different behaviours of mutant products reported in previous cell-based studies (Vernes et al., 2006). In addition, our findings add to the described deficits in motor-skill learning and synaptic plasticity ob-

served in R552H heterozygous mice (Groszer et al., 2008). It is important to be cautious when extrapolating from mouse studies to consideration of human disorder, particularly in relation to speech and language impairments (Fisher and Scharff, 2009); for example, the differences in high-frequency sensitivity we observed are in a range (when appropriately transposed to the human situation) that is unlikely to have relevance for speech perception (Ehret and Kurt, in press). Nevertheless, the alterations of other ABR parameters in the R552H mice raise the intriguing possibility that humans with certain types of heterozygous FOXP2 mutations may show alterations in auditory processing which potentially impact on orofacial motor-skill learning and individual strategies for speech therapy. Future studies of human cases are warranted in order to assess this hypothesis.

4. Experimental procedures

4.1. Animals

Generation and initial characterization of the *Foxp2* mouse mutants used in this study were previously described by Groszer et al. (2008). Animals tested were heterozygous for either the R552H or the S321X point mutation. Mutants were backcrossed onto a C3H/HeN genetic background prior to carrying out the phenotypic investigations described below. Comparisons were made to wildtype (WT) littermate controls, also on a C3H/HeN background. All animals were tested when they were between 50 and 61 days old. In total, data were obtained from 6 heterozygous R552H animals and 8 C3H/HeN WT matched littermates, as well as from 7 heterozygous S321X animals and 8 C3H/HeN WT matched littermates, including both sexes. The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the appropriate authority (Regierungspräsidium Tübingen, Germany).

4.2. ABR measurements

ABRs to pure tones (2–45.25 kHz, 2 steps/octave) were recorded in anesthetized animals in a sound attenuating chamber (IAC, Industrial Acoustics Company). Anesthesia was performed by intraperitoneal injection of a mixture of 100 mg/kg ketamin hydrochloride (Ketavet 100, Pharmacia), 15 mg/kg xylazin hydrochloride (Rompun 290, Bayer) and 0.5 mg/kg atropin sulfate. Supplemental doses of anesthetics were given subcutaneously as needed to keep the animals motionless during the recording sessions. During recording, the animals were placed on a feedback controlled heating pad for keeping their body temperature constant at 37 °C.

Generation of acoustic stimuli and recording of evoked potentials were performed using a Multi IO Card (National Instruments, E-6052). Tone bursts (12 ms duration, 1 ms rise and fall time, cosine-shaped) were delivered free-field at a rate of 3/s through a dynamic speaker (Beyer DT911). Sound pressure levels (SPLs) were measured and calibrated at the animal's ear with a 6.35 mm probe microphone (Brüel & Kjaer 4135, Naerum, Denmark) and a measuring amplifier (Brüel & Kjaer 2610). The synthesized tones were adjusted in level to

generate a flat ± 6 dB sound field at the animal's ear in the frequency range tested. Distortion products were at least 25 dB below the tone levels at the highest levels (100 dB) used. To record bioelectrical potentials, subdermal silver wire electrodes (diameter 0.25 mm, Goodfellow) were inserted at the vertex (reference), ventrolateral to the left ear (active) and dorso-sacrum at the back of the animal (ground). After amplification (100 dB) and bandpass filtering (200 Hz to 5 kHz), electrical signals were averaged over 256 tone burst repetitions. The custom made software included an artifact rejection code (all waveforms with a peak to peak amplitude exceeding a defined voltage were rejected) to eliminate heart beat and muscle activity. The stimulus intensities used ranged from 20 to 100 dB SPL (5 dB steps).

4.3. Data analysis

The ABR hearing threshold was determined as the lowest SPL that produced the characteristic waveform of the ABR potentials visually distinct from the noise level (Knipper et al., 2000). Threshold values of ABRs were compared between heterozygous mutants and WT littermates for statistically significant differences by means of Student's two-tailed t-test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) and arranged across frequencies to obtain audiograms for each animal group. To further characterize ABR responses we analyzed peak latencies and peak-to-trough amplitudes for all five ABR peaks (I to V). We then tested for differences between heterozygotes and WT animals with Student's two-tailed t-test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). From average ABR waveforms, differences between heterozygotes and WTs (value of heterozygote minus value of WT) were calculated for peak amplitudes and peak latencies at each frequency tested. These differences were then averaged across all stimulation frequencies and tested with a one sample t-test against the expected value of zero difference ($*P < 0.05$, $**P < 0.01$, $***P < 0.01$).

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