11) *In(3L)68F;70C*, unique endemic: Rio de Janeiro, RJ, 2004. (1)
12) *In(3R)84D;98F*, Figure 3-e, unique endemic: Rio de Janeiro, RJ, 2005. (1)
13) *In(3R)87F;92F*, recurrent endemic: Rio de Janeiro, RJ, 2004; Florianópolis, SC, 2006. (2)
14) *In(3R)87E;98C*, Figure 3-j, recurrent endemic: Rio de Janeiro, RJ, 2004; Campinas, SP, 2004. (4)

References**:** Inoue, Y., and Y. Igarashi 1994, Jpn. J. Genet. 69: 105-118; Keller, A., 2007, Curr. Biol. 17: 77-81; Krimbas, C.B., and J.R. Powell 1992, *Drosophila Inversion Polymorphism* (Krimbas, C.B., and J.R. Powell, eds.), pp. 1–52, Boca Raton, CRC Press; Lemeunier, F., and S. Aulard 1992, *Drosophila Inversion Polymorphism* (Krimbas, C.B., and J.R. Powell, eds.), pp. 339–405, Boca Raton, CRC Press; Powell, J.R., 1997, *Progress and Prospects in Evolutionary Biology, The Drosophila Model* (Powell, J.R., ed.), New York, Oxford University Press; Mettler, L.E., R.A. Voelker, and T. Mukai 1977, Genetics 87: 169-176.

Hearing defects in Johnston's Organ Gal4 lines.

Kotla, Sumankrishna[1], Maura Cosetti[2], Peter F. O'Brien[1], and Frances Hannan[1].
[1]Departments of Cell Biology & Anatomy, and Otolaryngology, New York Medical College, Valhalla, NY 10598; [2]Department of Otolaryngology, New York Eye & Ear Infirmary, New York, NY 10003; Email: frances_hannan@nymc.edu.

Introduction

The *Drosophila* auditory organ, Johnston's Organ (JO), is housed in the second antennal segment (a2; Caldwell and Eberl, 2002). It consists of an array of more than 200 mechanosensory chordotonal organs termed scolopidia. Each scolopidium contains two to three bipolar neurons, and a number of support cells including the scolopale cell which ensheaths the ciliated dendritic processes of the neurons. The basal ends of the scolopidia are attached to the inner surface of a2, while the apical ends are attached to the joint between the a2 and a3 segments by a dendritic cap that is secreted by the scolopale cell and possibly other support cells. The vibrating air particles of a near-field sound, typically the flies' courtship song, cause deflection of the arista and rotation of the third antennal segment (a3) about the a2/a3 joint. This stretches the entire array of scolopidia, initiating transmission of a signal to the central brain via the antennal nerve.

A number of Gal4 lines that label specific subsets of neurons within the JO have been identified (Sharma *et al*., 2002; Kamikouchi *et al*., 2006). The JO1 line labels most neurons in the JO (94%), while the JO3 line labels 67% of JO neurons. Three other lines label 22-38% of the JO neurons (JO2, JO4, JO15). An additional twelve lines label JO neurons as well as other cells within the antenna and forehead region (JO21-JO32). All of the lines also label cells elsewhere in the fly brain, except JO15, which expresses Gal4 only in the JO (Sharma *et al*., 2002). The JO15 line expresses Gal4 under control of a JO specific enhancer fragment, originally identified and cloned from a *hobo* enhancer trap line that specifically stains the JO neurons (Sharma *et al*., 2002).

The spatial organization of the JO neurons expressing Gal4 in each line has been determined, as well as their projection patterns to the antennal mechanosensory and motor center, the AMMC (Kamikouchi *et al*., 2006). Within the JO, the cell bodies of the JO neurons form a bottomless bowl shape. The JO3 line labels cells throughout the entire bowl region, while the JO2 line labels a middle

ring of cells, and the JO4 and JO15 lines each label clusters of cells in the anterior and posterior regions. Five distinct zones within the central brain receive projections from the JO neurons, four of which are within the AMMC, while the other extends over the ventrolateral protocerebrum and the subesophageal ganglion.

We are interested in using these lines to drive the expression of genes of interest and RNAi constructs in the JO to assess the effect of those constructs on hearing. Prior to using the JO lines for this purpose, however, we needed to determine whether any of the JO-Gal4 insertions disrupt hearing. We present data here showing that many of these JO-Gal4 lines do indeed have defective hearing, even after extensive outcrossing to wild type flies for five generations. Both dominant and recessive effects on hearing are observed, potentially identifying novel hearing genes. We also detected several JO-Gal4 lines that express strongly in the JO but do not affect hearing. These will be useful for our future studies and for other *Drosophila* hearing researchers.

Materials and Methods

Fly Stocks:

JO15/TM3Sb contains a pPTGAL element with a JO specific enhancer (Sharma *et al*., 2002). The remaining JO-Gal4 lines; JO1, JO2, JO3/CyO, JO4, JO21, JO22, JO23, JO24, JO25/+, JO26/CyO, JO27/CyO, JO28, JO29, JO30, and JO31, were identified by Kamikouchi *et al*. (2006) from the NP series of lines produced by mobilization of pGawB in a *y w (iso)* background (Yoshihara and Ito, 2000). The isogenic control line used in this study is $w^{118isoCJ}$ (Yin *et al*., 1994). Flies were raised on a cornmeal medium at 25°C, and females were collected from 2-4 days post-eclosion for electrophysiological recording of Sound Evoked Potentials (SEPs).

Electrophysiology:

Unanesthetized flies were mounted into a 200 μl micropipette tip with the protruding head immobilized by modeling wax, leaving the antennae free to vibrate. A sharp tungsten electrode was placed in the joint between the first and second antennal segments (recording electrode), and a second (reference) electrode was placed in the brain in the dorsal medial region. The electrodes were connected to a DP-301 differential amplifier (Warner Instruments, CT) with gain set at 1000, low pass filter at 10kHz and high pass filter at 10Hz. The sound stimulus consisting of 5 pulses with 35ms interpulse interval (mimicking the pulse component of *Drosophila* courtship song) was delivered from a speaker via a 0.25 inch i.d. tubing to ensure near field acoustic conditions. Signals were acquired and digitized using a PCI-6023E data acquisition board, and Lab View software, Version 8.0 (National Instruments, Austin, TX). The entire apparatus was set up in a Faraday cage to eliminate electrical disturbances. Responses to 10 presentations of the stimulus were averaged in each trial, and the maximum amplitude of the peak responses was calculated. Statistical analyses were performed using Prism software, Versions 4.0c/5.00 (Graph Pad, San Diego, CA).

Results and Discussion

Previous studies in our lab have shown that hearing in female flies is more sensitive to disruption than male hearing (Cosetti *et al*., submitted); therefore, we restricted this study to female flies. We first recorded SEPs from all the JO-Gal4 lines, and compared them with recordings from the $w^{118isoCJ}$ isogenic control line that is wild type for hearing, and is hereafter referred to as WT. This line is commonly used for behavioral studies (Yin *et al*., 1994) and is similar to the lines used to generate the transgenic JO-Gal4 insertions (see Methods). The peak amplitudes of the SEPs in

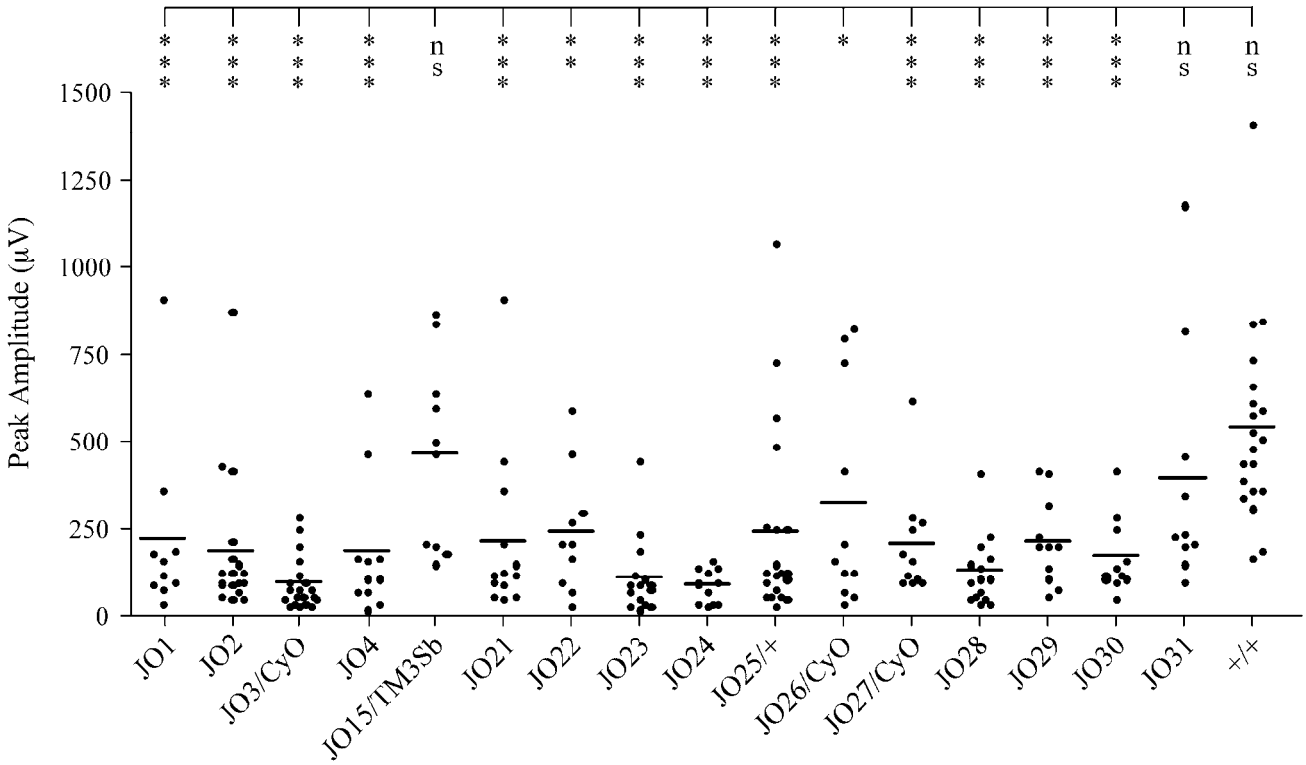


Figure 1. Peak amplitudes of SEPs in JO-Gal4 lines are reduced in comparison to wild type controls. The majority of flies with JO-Gal4 insertions exhibit SEP responses ranging from ~20-500 µV, that are much lower than wild type SEPs. Representative responses of wild type (+/+) flies range from ~100-1500 µV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded wild type (WT) dataset (n = 80) is shown as a horizontal dashed line. Statistically significant reductions in median SEPs are observed for most JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; ** $p < 0.005$; ***$p < 0.005$; Mann-Whitney). See Table 1 for detailed statistical analyses.

individual flies of each genotype, including a representative group of contemporaneously recorded WT flies (+/+), are plotted graphically in Figure 1. The peak amplitude of the response in wild type females ranges from 100 µV up to 1500 µV, while many of the JO lines exhibited responses below 100 µV, especially JO3, JO23, JO24 and JO28. The JO3, JO15, JO26 and JO27 lines were supplied as balanced stocks; however, the presence of the CyO or TM3 balancer chromosomes had no effect on SEPs (Figure 1; data not shown). The mean response for each genotype is shown as a horizontal bar, while the mean response of a large WT dataset (n = 80), collected over several months of recordings, is shown as a horizontal dashed line (Figure 1). Statistical analyses of the data, including minimum, maximum, median, mean and standard error of the mean (SEM) are presented in Table 1. P values were calculated by non-parametric Mann-Whitney analysis of median values for each group in comparison to the large WT dataset (Table 1). Significant reductions in median peak response amplitude were observed for most of the JO-Gal4 lines when compared to WT flies (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; Figure 1), except for the JO15/TM3Sb and JO31lines (ns $p > 0.05$; Figure 1).

Table 1. Statistical evaluation of SEPs in JO-Gal4 lines.

Genotype	N	Minimum (mV)	Maximum (mV)	Median (mV)	Mean (mV)	SEM (mV)	P value vs WT
WT	80	110	1410	525	509	30	1
JO1	10	40	910	140	223	81	0.0005
JO2	18	50	880	115	187	48	<0.0001
JO3/CyO	20	30	290	70	96	17	<0.0001
JO4	12	20	640	135	183	53	<0.0001
JO15/TM3Sb	10	150	870	485	466	86	0.7531
JO21	13	50	910	120	216	67	<0.0001
JO22	10	30	590	210	242	56	0.0016
JO23	15	20	450	90	113	29	<0.0001
JO24	12	30	160	95	90	13	<0.0001
JO25/+	20	30	1070	130	242	61	<0.0001
JO26/CyO	11	40	830	160	326	95	0.0440
JO27/CyO	11	100	620	160	209	46	0.0001
JO28	15	40	410	110	129	25	<0.0001
JO29	11	60	420	200	216	37	0.0002
JO30	11	50	420	120	170	32	<0.0001
JO31	10	100	1180	235	394	109	0.0744
+/+	20	170	1410	495	540	62	0.7271

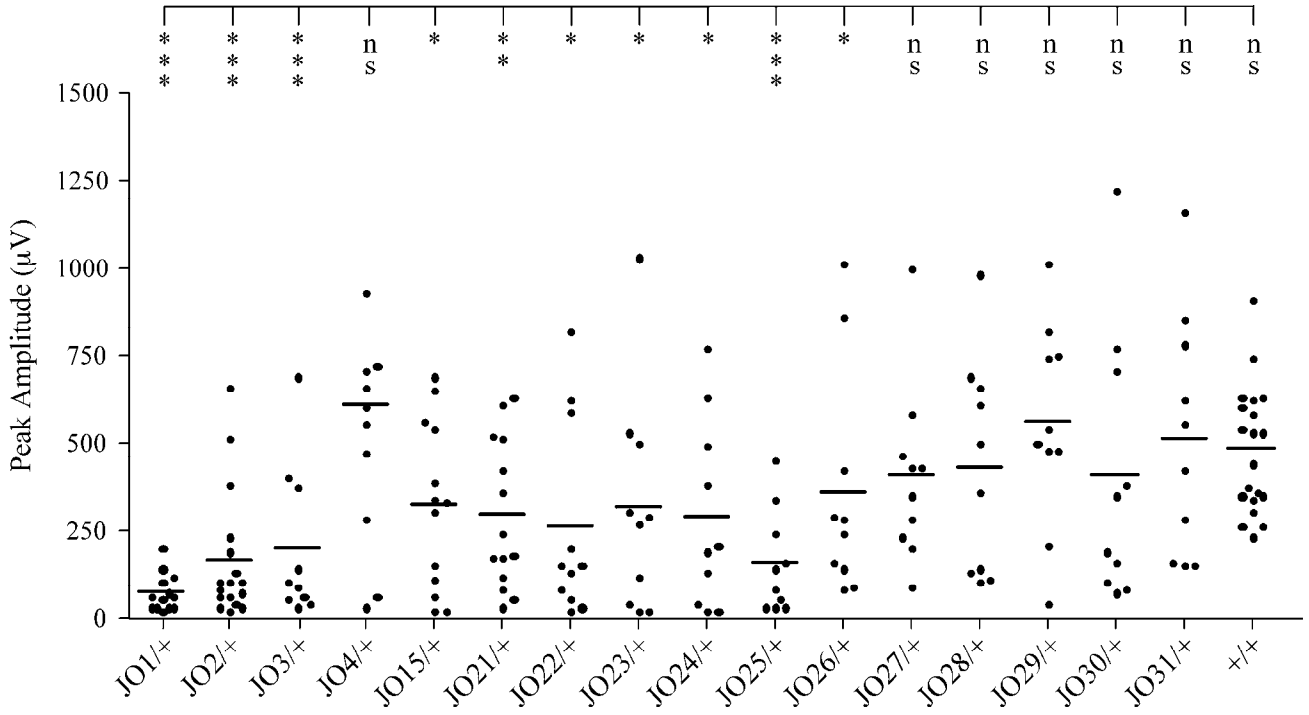


Figure 2. Peak amplitudes of SEPs are improved in JO-Gal4 heterozygotes. Many JO-Gal4 heterozygotes exhibit SEP responses that are statistically similar to wild type values (ns $p > 0.05$), ranging from ~50-1200 µV. Representative responses of wild type (+/+) flies range from ~200-950 µV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded WT dataset (n = 80) is shown as a horizontal dashed line. Statistically significant

reductions in median SEPs are still observed for some JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; ** $p < 0.005$; ***$p < 0.005$; Mann-Whitney). See Table 2 for detailed statistical analyses.

Table 2. Statistical evaluation of SEPs in JO-Gal4 heterozygotes.

Genotype	N	Minimum (mV)	Maximum (mV)	Median (mV)	Mean (mV)	SEM (mV)	P value vs WT
JO1/+	14	20	200	60	71	14	<0.0001
JO2/+	17	20	660	100	164	45	<0.0001
JO3/+	10	30	690	95	197	69	0.0005
JO4/+	11	30	1650	600	606	134	0.4252
JO15/+	13	20	690	330	320	66	0.0212
JO21/+	14	30	630	210	292	57	0.0048
JO22/+	11	20	820	150	258	84	0.0060
JO23/+	10	20	1030	280	312	99	0.0145
JO24/+	10	20	770	200	288	85	0.0251
JO25/+	10	30	450	110	155	46	<0.0001
JO26/+	10	80	1010	260	357	102	0.0419
JO27/+	10	90	1000	390	405	80	0.1542
JO28/+	10	100	980	430	428	97	0.4411
JO29/+	10	40	1010	520	557	91	0.4526
JO30/+	10	70	1220	270	403	121	0.1382
JO31/+	10	150	1160	485	512	109	0.9590
+/+	20	230	910	485	479	41	0.7728

Since the JO-Gal4 lines would be used as heterozygotes when driving expression of transgenic constructs, we next analyzed the responses of heterozygous JO-Gal4 flies, after crossing the JO-Gal4 lines to the WT line, as shown in Figure 2. Statistical analyses of the data are presented in Table 2. Hearing was not significantly different from wild type flies in JO4/+, JO27/+, JO28/+, JO29/+, JO30/+ and J031/+ flies (ns $p > 0.05$; Figure 2). There also appeared to be an improvement of SEPs in JO21/+, JO22/+, JO23/+, and JO24/+ flies (* $p < 0.05$; **$p < 0.005$; Figure 2) as compared to JO21, JO22, JO23, and JO24 homozygotes (**$p < 0.005$; ***$p < 0.0005$; Figure 1); however, this "improvement" was not statistically significant ($p > 0.05$; not shown). Several lines, however, were still significantly unresponsive to the stimulus, including JO1/+, JO2/+, JO3/+ and JO25/+ (***$p < 0.005$, Figure 2). Unfortunately three of these lines are among those that express strongly in the JO region of the antenna alone (Kamikouchi *et al*., 2006), and would, therefore, be particularly useful for expressing transgenic RNAi constructs in the JO.

Encouraged by the restoration of normal hearing in some of the JO-Gal4 heterozygotes, we continued to outcross to the WT line for an additional four generations, which should be sufficient to remove any effect of genetic background differences on hearing. The outcrossed JO-Gal4 lines are hereafter distinguished from the original JO-Gal4 lines by a $^{\#}$ superscript. Lines were then made homozygous (except JO15$^{\#}$/TM3 Ser, and JO27$^{\#}$/CyO, which are homozygous lethal/semi-lethal) and SEPs were again recorded and compared to wild type flies, as shown in Figure 3 and Table 3. Significant hearing defects were still observed in the majority of homozygous lines, including JO1$^{\#}$, JO2$^{\#}$, JO3$^{\#}$, JO22$^{\#}$, JO23$^{\#}$, JO24$^{\#}$, JO25$^{\#}$, JO26$^{\#}$, JO28$^{\#}$, and JO31$^{\#}$ (**$p < 0.005$; ***$p < 0.0005$; Figure 3). The SEPs of JO4$^{\#}$, JO15$^{\#}$/TM3 Ser, JO27$^{\#}$/CyO, and JO30$^{\#}$ lines were, however, indistinguishable from wild type flies (ns $p > 0.05$; Figure 3). The apparent improvement of SEPs in JO21$^{\#}$ and JO29$^{\#}$ homozygotes (* $p < 0.05$; Figure 3) compared to the original JO21 and JO29 flies (*** $p < 0.0005$; see Figure 1) was not statistically significant ($p > 0.05$; not shown).

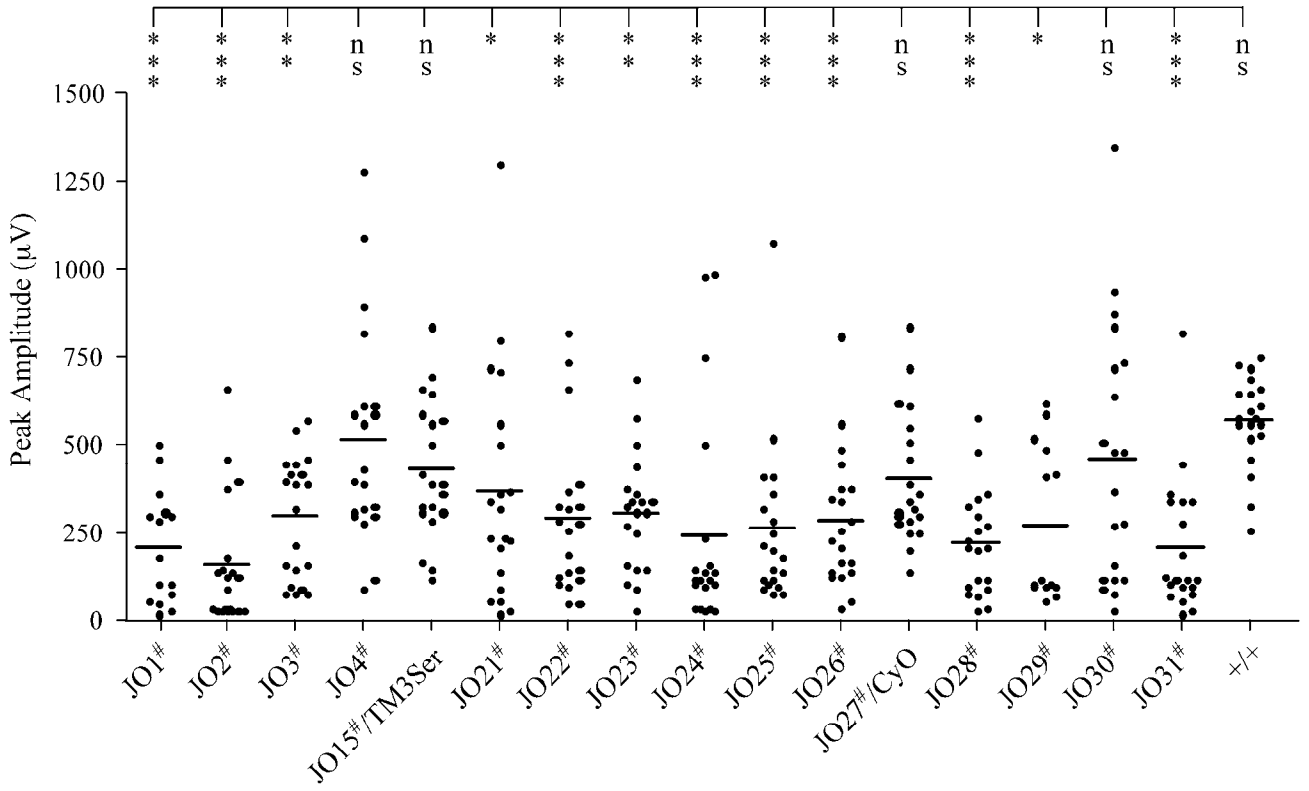


Figure 3. Peak amplitudes of SEPs in most JO-Gal4 lines remain reduced after extensive outcrossing to wild type flies. The majority of flies with JO-Gal4 insertions exhibit reduced SEP responses, ranging from ~20-800 μV, despite extensive outcrossing to wild type controls. Representative responses of wild type (+/+) flies range from ~250-750 μV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded wild type (WT) dataset (n = 80) is shown as a horizontal dashed line. Statistically significant reductions in median SEPs are observed for many JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; ** $p < 0.005$; ***$p < 0.005$; Mann-Whitney). See Table 3 for detailed statistical analyses.

We then analyzed the response of heterozygous outcrossed JO-Gal4 lines, after crossing to the WT line, as shown in Figure 4 and Table 4. The majority of the outcrossed JO-Gal4 heterozygotes exhibit SEPs that are statistically indistinguishable in comparison to wild type flies (ns $p > 0.05$; Figure 4). A few of these lines still show significantly reduced SEPs compared to WT flies, however, suggesting a dominant effect of the Gal4 insertion on hearing in these flies, including JO3$^{\#}$/+, JO26$^{\#}$/+, JO27$^{\#}$/+ and JO31$^{\#}$/+ flies (*$p < 0.05$, Figure 4). The SEP defects in these four JO-Gal4 lines may improve further after additional outcrossing; however, we have decided not to use these lines to drive expression of genes in the JO for the analysis of hearing. Interestingly, the JO27/+ and JO27$^{\#}$/CyO flies appear to have normal hearing (Figures 2 and 3), while the JO27$^{\#}$/+ flies do not (Figure 4). Perhaps there is an interaction with another gene or genes on the WT or CyO second chromosomes.

The dominant defect in SEPs in the JO3, JO26, JO27 and JO31 lines (Figure 4), suggests that the genes disrupted by these JO-Gal4 insertions may be critical for hearing, although this needs to be confirmed by additional experiments. The JO3 line is expressed strongly in the JO alone and has an insertion in the CG13795 gene, encoding a putative extracellular amino acid transporter protein,

Table 3. Statistical evaluation of SEPs in outcrossed JO-Gal4 lines.

Genotype	N	Minimum (mV)	Maximum (mV)	Median (mV)	Mean (mV)	SEM (mV)	P value vs WT
JO1$^{\#}$	15	20	500	180	211	41	<0.0001
JO2$^{\#}$	20	30	660	110	158	40	<0.0001
JO3$^{\#}$	20	80	570	355	297	38	0.0007
JO4$^{\#}$	20	90	1280	415	516	69	0.7630
JO15$^{\#}$/TM3Ser	20	120	840	390	432	44	0.2776
JO21$^{\#}$	20	20	1300	280	365	72	0.0133
JO22$^{\#}$	20	50	820	270	292	50	0.0005
JO23$^{\#}$	20	30	690	320	307	37	0.0007
JO24$^{\#}$	20	30	990	120	246	68	<0.0001
JO25$^{\#}$	20	80	1080	190	262	52	<0.0001
JO26$^{\#}$	20	40	810	245	287	42	0.0004
JO27$^{\#}$/CyO	20	140	840	330	402	41	0.0738
JO28$^{\#}$	20	30	580	210	222	33	<0.0001
JO29$^{\#}$	14	60	620	115	273	58	0.0013
JO30$^{\#}$	20	30	1350	425	461	81	0.3476
JO31$^{\#}$	20	20	820	120	209	43	<0.0001
+/+	20	260	750	580	571	29	0.0963

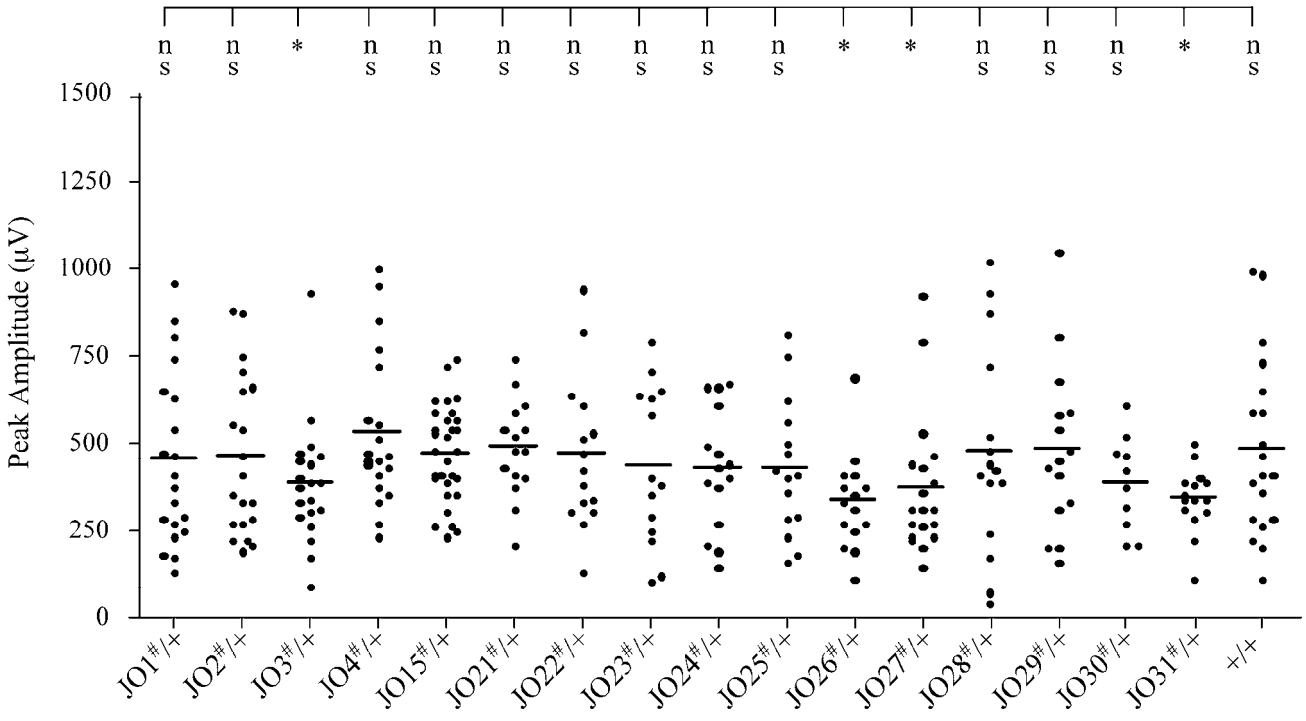


Figure 4. Peak amplitudes of SEPs in outcrossed JO-Gal4 heterozygotes are restored to wild type values. The majority of outcrossed JO-Gal4 heterozygotes exhibit SEP responses that are statistically similar to wild type values (ns $p > 0.05$), ranging from ~100-1000 µV. Representative responses of wild type (+/+) flies also range from ~100-1000 µV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded wild type (WT) dataset (n = 80) is shown as a horizontal dashed line. Statistically significant reductions in median SEPs are observed for only

a few JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; Mann-Whitney). See Table 4 for detailed statistical analyses.

Table 4. Statistical evaluation of SEPs in outcrossed JO-Gal4 heterozygotes.

Genotype	N	Minimum (mV)	Maximum (mV)	Median (mV)	Mean (mV)	SEM (mV)	P value vs WT
JO1#/+	20	130	960	390	451	55	0.4081
JO2#/+	20	190	880	380	458	51	0.4879
JO3#/+	20	90	930	380	384	39	0.0277
JO4#/+	20	230	1000	455	529	49	0.7662
JO15#/+	30	230	740	480	472	25	0.6798
JO21#/+	15	210	740	480	487	36	0.9878
JO22#/+	15	130	940	420	466	56	0.5302
JO23#/+	14	100	790	390	436	61	0.4967
JO24#/+	15	140	670	430	427	45	0.4230
JO25#/+	15	160	810	410	429	50	0.3197
JO26#/+	15	110	690	330	332	35	0.0089
JO27#/+	19	140	920	310	372	46	0.0193
JO28#/+	15	40	1020	420	474	77	0.5921
JO29#/+	15	160	1050	450	481	62	0.7019
JO30#/+	10	210	610	395	386	42	0.1451
JO31#/+	15	110	500	340	341	24	0.0079
+/+	19	110	990	410	484	58	0.7390

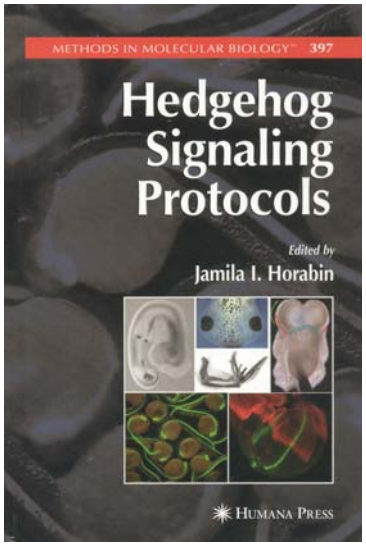
while JO26 is downstream of the Hr38 hormone receptor-like gene, JO27 is upstream of the CG17834 gene, encoding a hypothetical protein of unknown function, and JO31 disrupts a glucuronosyltransferase transcript encoded by CG17323 (Kamikouchi *et al*., 2006). The Gal4 insertions in the JO1 and JO2 lines are expressed strongly in the JO alone, and also appear to affect genes that are required for hearing, since our manipulations failed to restore SEPs in JO1 or JO2 homozygous flies (Figure 3). The insertion site in the JO1 line is unknown; however, the JO2 insertion is located in the first intron of the polyhomeotic proximal (ph-p) gene (Kamikouchi *et al*., 2006), that is involved in regulation of the bithorax complex and is required for CNS development, among other things. Conversely, while the Gal4 insertions in the JO4 and JO15 lines are also expressed strongly in the JO, these insertions do not appear to affect hearing. The JO4 insertion is reported to be downstream of a putative gene of unknown function (CG40138; Kamikouchi *et al*., 2006). The JO15 insertion causes a homozygous lethal phenotype; however, the Gal4 expression pattern should be independent of the insertion site, since it is determined by an enhancer element within the pPTGAL construct (Sharma *et al*., 2002).

It is tempting to speculate that the subset of JO neurons labeled by JO2 and JO3 are somehow specialized for hearing, while those labeled by JO4, and perhaps JO15, have some other function such as hygrosensation, or detection of gravity or acceleration. For our future studies we will be able to utilize the JO1#, JO2#, JO4# and JO15# lines, which exhibit no hearing defects in heterozygous flies, to drive expression of our UAS linked constructs in substantial numbers of JO neurons, while not affecting other types of neurons in the antenna. It is also possible that the JO1 and JO2 heterozygotes will be sensitized for hearing defects, fostering identification of subtle hearing defects caused by expression of our constructs. Many of the other JO lines that do not affect hearing in heterozygotes, including JO21#, JO22#, JO23#, JO24#, JO25#, JO28#, JO29# and JO30#, may also prove useful for expressing constructs in the JO, depending on their expression in other brain areas.

Acknowledgments: We thank Dan Eberl for the JO15 line and helpful advice regarding our electrophysiology setup, and Asuza Kamikouchi for providing the remaining JO lines. We also thank Dr. Guopei Yu for advice regarding the statistical analysis of data, and Dr. Steven D. Schaefer MD, FACS for his enthusiastic support and encouragement. This work was supported by funds from the New York Eye & Ear Infirmary, New York, NY; and the Children's Hearing Foundation, New York, NY.

References: Caldwell, J.C., and D.F. Eberl 2002, J. Neurobiol. 53: 172-189; Cosetti, M.K., D. Culang, S. Kotla, P.F. O'Brien, D.F. Eberl, and F. Hannan A unique transgenic model for hearing loss (submitted); Kamikouchi, A., T. Shimada, and K. Ito 2006, J. Comp. Neurol. 499: 317-356; Sharma, Y., U. Cheung, E.W. Larson, and D.F. Eberl 2002, Genesis 34: 115-118; Yin, J.C., J.S. Wallach, M. Del Vecchio, E.L. Wilder, H. Zhou, W.G. Quinn, and T. Tully 1994, Cell 79: 49-58; Yoshihara, M., and K. Ito 2000, Dros. Inf. Serv. 83: 199-202.

New Books



Hedgehog Signaling Protocols.

Horabin, Jamila I. (editor). 2007. *Hedgehog Signaling Protocols*. Methods in Molecular Biology #397. Humana Press, Totowa, NJ. 256 pp. ISBN: 978-1-58829-692-4.

The Hedgehog signaling pathway is important to understanding the regulation of development in both invertebrates and vertebrates. Its misregulation is associated with many human diseases. This well-organized book presents several different experimental approaches toward understanding its molecular events in a variety of model systems. Protocols are presented in a clear manner with excellent illustrations of techniques, experimental resources, and representative results. In 16 chapters, the 38 contributors provide a valuable resource for geneticists, biochemists, and molecular biologists studying this important pathway. Following a description of Hedgehog protein purification, applications in several model systems are discussed, including the chick limb, *Xenopus*, rats, zebrafish, and *Drosophila*. Specific techniques include manipulating Hedgehog signaling with retroviral expression systems, cell cycle analysis using flow cytometry, detecting tagged Hedgehog with immunocytochemistry, confocal analysis, RNAi, germline clone analysis, clonal analysis in somatic tissues, GAL4/UAS targeted gene expression, biochemical fractionation of cells, immunoprecipitation to study protein-protein interactions, and gene sequence analyses. Each chapter is accompanied by a useful and up-to-date bibliography of cited references. Price: $99.50. For more information, email humana@humanapr.com.