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16. The plasmid pFL-EBOVe$^+$ was designed to place the T7 promoter adjacent to the viral leader region, and the viral trailer region was constructed to be adjacent to a ribozyme sequence followed by tandem terminators of T7 transcription. In this case, the correct 3′ end of the transcribed EBOV antigenome, free of additional nucleotides, was generated by self-cleavage of the ribozyme (*24*). To increase the transcriptional activity of the T7 RNA polymerase, an additional guanosine residue was introduced between the promoter of the T7 polymerase and the first residue of the EBOV genome (*25*). The length of the encoded antigenome (FL-EBOVe$^+$) is therefore increased by 1 nt compared to that of the wild-type virus (GenBank accession number, AF086833). The size of the full-length antigenome encoded by the plasmid pFL-EBOVe$^-$ was 1 nt longer than that encoded by pFL-EBOVe$^+$ and 2 nt longer than the size of the genome of the wild-type virus because of the insertion of additional adenosine residues at the editing site. Mutations at the GP gene editing site were introduced into the plasmid pKSS25 by site-directed mutagenesis using the primers 5′-GG GAAACTAAGAA-GAAACCTCACTAG and 5′-CTAGTGAGGTTTCTTCT-TAGTTTCCC.
17. The Sal I restriction site (GTCGAC) located in the GP gene at position 6180 was mutated by site-directed mutagenesis using the pair primers 5′-GGTTAGTGAT-GTAGATAA ACTAGTTTG and 5′-CAAACTAGTTTATC TACATCACTAACC. This mutation is silent. In addition, an accidental mutation in a nontranslated region of the L gene (A → U at position 18227) was found after complete sequence analysis of the final plasmid clones.
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20. BSR T7/5 cells were grown overnight to about 60 to 80% confluency in 25-cm^2 flasks in 1× Glasgow medium supplemented with 10% NCS (newborn calf serum). One hour before transfection, cells were washed twice with medium without NCS. Cells were transfected with a plasmid mixture containing 2 μg of full-length plasmid (pFL-EBOVe+), 0.2 μg of pT/VP30EBOV, 0.5 μg of pT/VP35EBOV, 0.5 μg of pT/NPEBOV, and 1 μg of pGEM-LEBOV (*14, 19*). Transfection experiments were carried out with a Fusion 6 reagent (transfection protocol supplied by Roche). The transfection medium was removed at 8 hours after transfection; cells were washed and maintained in Glasgow medium containing 2.5% NCS for 6 to 9 days after transfection. On average, in each rescue experiment, approximately 100 foci of rounded cells were observed in the cell culture flask (about 1×10^5 to 2×10^5 cells). That means that one in approximately 10^3 cells allowed the formation of viral particles. However, virus release from BHK cells was extremely low, and amplification of recombinant EBOV on Vero cells was necessary before further analysis. Determination of the virus titers by plaque formation showed that about 200 infectious particles were recovered from the average rescue experiment.
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27. For electron microscopy, 72 hours after infection, control and virus-infected cells were fixed with Hanks' balanced salt solution (HBSS) containing 2.5% glutaraldehyde, postfixed with HBSS containing 1% osmium tetraoxide, dehydrated, and embedded in Epon. Ultrathin sections were cut, placed on 200-mesh copper electron microscopy grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss 109 transmission electron microscope at 80 kV.
28. See Web Fig. 2 on Science Online at www.sciencemag.org/cgi/content/full/1057269/DC1.
29. For immunofluorescent staining, Vero E6 cells were infected at a multiplicity of infection (MOI) of 10^{-2} and were processed 3 days later for indirect immunofluorescence analysis. Cells grown on coverslips were washed with phosphate-buffered saline (PBS) solution, fixed with 4% paraformaldehyde at 4°C for 24 hours, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The nonspecific binding was blocked by blocking buffer (2% bovine serum albumin, 5% glycerol, and 0.2% Tween-20 in PBS), and cells were then incubated with 100 mM glycine for 10 min, washed with PBS, and incubated at 4°C for 18 hours with the respective antibodies [human monoclonal antibody (mAb) KZ52, which is specific for the EBOV GP (dilution 1:100 in blocking buffer); and mouse mAb B6C5, which is specific to EBOV NP (dilution 1:10 in blocking buffer)]. Subsequently, cells were washed three times with PBS and stained with fluorescein isothiocyanate–conjugated goat anti-human immunoglobulin G (IgG) (diluted 1:50 in blocking buffer) or with rhodamine-conjugated goat anti-mouse IgG (1:100 in blocking buffer) for 1 hour at room temperature. Nuclear counterstaining was performed with 4′,6′-diamidino-2-phenylindole (DAPI) (0.1 μg/ml). Finally, cells were washed three times with PBS, dipped into dH2O, covered with mounting medium, coverslipped, and examined with a fluorescence microscope (Axiomat, Zeiss) with digital photographic equipment for taking images (Spot camera system, version 2.1.2, Diagnostic Instruments).
30. See Web Fig. 3 on Science Online at www.sciencemag.org/cgi/content/full/1057269/DC1.

31. All experiments involving infectious EBOV were carried out in biosafety level 4 (BSL4) laboratories at the Institute of Virology in Marburg, Germany, and at the Jean Merieux P4 Research Center in Lyon, France. We thank D. Burton and P. Parren for providing mAb KZ52A; S. Becker for mAb B6C5; K.-K. Conzelmann for the BSR T7/5 cell line; A. Sergeant and E. Derrington for critical reading of the manuscript; and C. Laukel and M. Rossi for expert technical help. M.W. was supported as a recipient of a fellowship from the Boehringer Ingelheim Fonds. This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 286), the Fonds der Chemischen Industrie, and INSERM; and by a grant from the Fondation pour la Recherche Medicale to V.E.V.

8 November 2000; accepted 22 January 2001
Published online 1 February 2001;
10.1126/science.1057269
Include this information when citing this paper.

Genetic Correlates of Musical Pitch Recognition in Humans

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We used a twin study to investigate the genetic and environmental contributions to differences in musical pitch perception abilities in humans. We administered a Distorted Tunes Test (DTT), which requires subjects to judge whether simple popular melodies contain notes with incorrect pitch, to 136 monozygotic twin pairs and 148 dizygotic twin pairs. The correlation of DTT scores between twins was estimated at 0.67 for monozygotic pairs and 0.44 for dizygotic pairs. Genetic model-fitting techniques supported an additive genetic model, with heritability estimated at 0.71 to 0.80, depending on how subjects were categorized, and with no effect of shared environment. DTT scores were only weakly correlated with measures of peripheral hearing. This suggests that variation in musical pitch recognition is primarily due to highly heritable differences in auditory functions not tested by conventional audiologic methods.

The perception of pitch requires both the ear, which receives auditory signals, and the brain, which performs substantial processing of auditory signals to produce a perceived pitch (*1–3*). Although the general features of human pitch processing have been well described, the precise cellular and molecular mechanisms involved remain largely obscure. One approach to understanding the mechanisms of pitch perception is to use genetic methods that exploit naturally occurring variation in pitch perception ability (*4*). If such variability is due to genetic factors, linkage and positional cloning studies could identify genes that encode the components of the pitch perception apparatus (*5*). To examine the genetic contributions to musical pitch

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recognition ability in humans, we performed a twin study (*6*) using the Distorted Tunes Test (DTT), which requires subjects to recognize notes with incorrect pitch in simple popular melodies (*7*).

The original DTT was developed in the 1940s (*8*) and used in large studies in the British population. These studies suggested that cultural biases and the effects of musical experience could be minimized by the appropriate choice of melodies, and that test scores in the same individual were stable across decades. They also revealed that a small but significant portion of the population (about 5%) scored no better than chance in their ability to distinguish correct from incorrect melodies. These individuals were classified as "tune deaf."

For our study, we created a similar, updated DTT and validated it for use in the current U.S. and British populations (*9*). The updated DTT was recorded on a compact disk and presented to all subjects in the same setting. Briefly, subjects were presented with 26 short popular melodies, ranging in length from 12 to 26 notes. Tunes were presented once, and after each presentation, subjects were asked to score whether the melody was correct or incorrect, and whether they were familiar or unfamiliar with that melody. We first measured the performance of 50 unrelated males and 50 unrelated females on the updated DTT. The distribution of scores in males and females did not differ (Kolmogorov-Smirnov = 0.94, Mann-Whitney test = 0.78). Test-retest scores in the same subject were highly correlated ($n = 40$, $r = 0.77$), confirming that like the original DTT, the updated DTT is reproducible in individuals. In contrast to results obtained by Kalmus and Fry with the original DTT, we did not observe a clear distinction between tune deaf and normal individuals.

Because our goal was to determine the extent to which genes and/or environment influence musical pitch-recognition ability, we chose a twin study, which can discriminate between the effect of the shared environment and that of shared genes. The study was approved by the St. Thomas' Hospital Research Ethics Committee (EC95/041, modification approved 29 September 1999), and informed consent was obtained from all subjects. A total of 284 female Caucasian twin pairs [136 identical (monozygotic, MZ) and 148 nonidentical (dizygotic, DZ)] aged 18 to 74 years from the St. Thomas' UK Adult Twin Registry (*10*) participated in the study. Subjects in this registry were ascertained from the general population through national media campaigns in the United Kingdom. Participating twins were part of an ongoing study into the genetics of common complex diseases (*10–13*). Twins were unaware of the specific hypotheses tested and were not screened for IQ, musical training, or musical experience.

The median ages of the MZ group and the DZ group were 50.7 and 47.9 years, respectively. Zygosity was determined by standardized questionnaire, with DNA fingerprinting used for confirmation (*10*). Each subject was administered the DTT and the 5 Minute Hearing Test (FMHT) to help identify subjects with potentially confounding hearing loss. The FMHT, promulgated by the American Academy of Otolaryngology, has been widely used for initial screening for hearing loss, and high correlations have been reported with a wide range of hearing measures, including pure-tone audiometry (*14*).

Using the DTT, we measured musical pitch recognition ability on an ordinal scale, scored as the number of correctly classified tunes. The scores of the subjects ranged from 26 (a perfect score) to 9. The distribution of scores in the MZ and DZ pairs is shown in Fig. 1, along with the overall score distribution of the entire sample. Although there was a trend for the MZ twins to have lower scores, this difference was not statistically significant (chi-square, $P = 0.12$; Kolmogorov-Smirnov, $P = 0.26$).

The statistical package STATA (*15*) was used to analyze the data. Spearman correlations were used to describe the associations between the scores on the DTT and the FMHT. A slight negative correlation between the DTT and the FMHT was observed (Spearman's $r = -0.10$, $P = 0.01$). Despite having statistical significance, this small negative correlation may or may not have functional significance. To test if this affected scores on the DTT, we divided subjects into a hearing and a hearing-impaired group on the basis of their FMHT scores (hearing impaired = FMHT score >15). No difference in DTT score was found between the groups as determined with statistical methods that account for the nonindependence of the twin pairs (generalized estimating equation, $P = 0.45$). There was no correlation between the DTT and age (Spearman's $r = -0.01$, $P = 0.87$). Probandwise concordance rates for the data divided into the two original categories described by Kalmus and Fry were 0.75 for MZ and 0.57 for DZ twin pairs, and a test for the difference was significant ($P = 0.01$) (*16*), indicating a genetic influence.

We applied genetic model-fitting techniques using the structural equation modeling package Mx (*17*) to obtain estimates of the genetic and environmental factors. Model fitting allows separation of the observed phenotypic variance into additive (A) or dominant (D) genetic components and common (C) and unique (E) environment. E also contains measurement error. The heritability, which estimates the extent to which variation in liability to disease in a population can be explained by genetic variation, can be defined as the ratio of genetic variance (A + D)

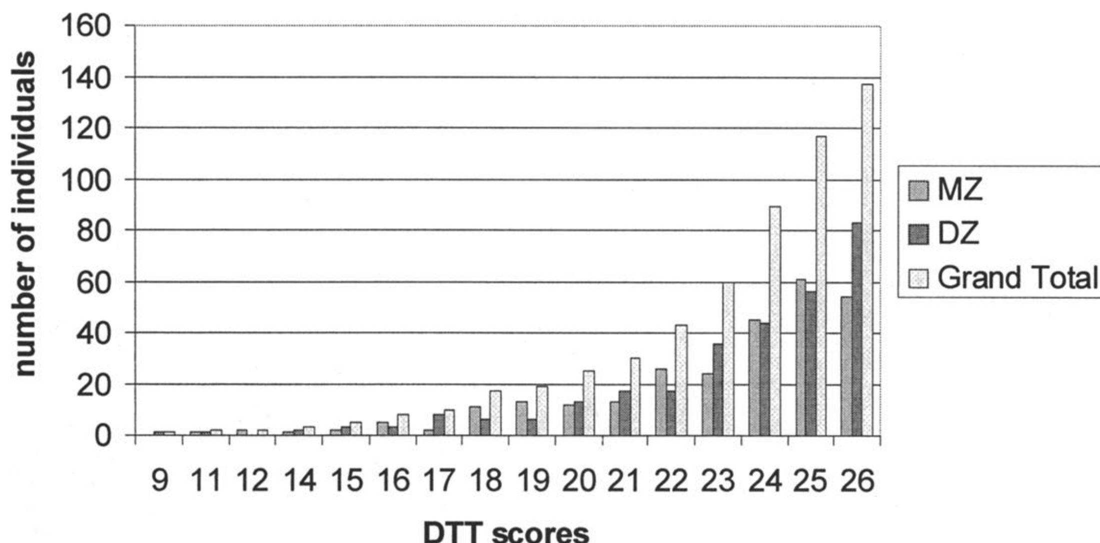

Fig. 1. Distribution of twin scores on the Distorted Tunes Test. Bars indicate the number of subjects attaining each score on the DTT. MZ, scores of monozygotic twins; DZ, scores of dizygotic twins.

to total phenotypic variance (A + C + D + E). We tested the significance of A, C, and D by removing them sequentially in specific submodels and testing the deterioration in model fit after each component was dropped from the full model. This leads to a model explaining the data with as few parameters as possible. Standard hierarchical chi-squared tests were used to select the best-fitting model (*18*).

The genetic and environmental contributions for categorical traits can be quantified by assuming a continuous underlying normal liability distribution with multiple thresholds that discriminate between the categories (*18*). We estimated the correlation in liability within the twin pairs by deriving polychoric correlations from the pairwise categorical distribution. The model-fitting approach compares the size of the polychoric correlations in MZ and DZ twins and provides estimates of the relative contribution of genetic and environmental factors to the liability distribution underlying musical pitch perception ability (*18*, *20*). Using the original definition of Kalmus and Fry (tune deaf = a score ≤23) on our data resulted in a large percentage of tune deaf individuals (39.6%). Using the updated DTT, we also did not observe a clear distinction between tune deaf and normal individuals. We therefore also fitted the data without imposing any arbitrary cut-off points and assigned categories as the raw scores themselves, with the exception of the few lowest scorers (scores between 9 and 15) that were assigned to one category, resulting in 12 categories. Contingency tables for the 12 categories were produced for the MZ and DZ twins and used as input data for Mx.

Analysis of the complete data (with no arbitrary cut-off point) showed a correlation in liability within the twin pairs of 0.67 and 0.44 for MZ and DZ twins, respectively (see Table 1, Model 1 for twin correlations in liability on the basis of two category data).

Across the analyses, the model providing the best fit to the data included an additive genetic and a unique environmental component. The heritability as estimated by genetic model-fitting with all of the available data was 71% [95% confidence interval (CI): 61 to 78%). Using the original cut-off value of Kalmus and Fry (≤23) to define two classes corresponds to a simplified model that contains only two groups: those with normal pitch recognition and those with some deficit in pitch recognition, regardless of severity. Using this model, we estimated heritability at 80% (95% CI: 65 to 90%). In both analyses, no dominant genetic effects and no significant effect of shared environment were detected (Table 1).

Table 1. Twin correlations and heritabilities of best-fitting models for the DTT data with two categories (Model I) or the actual scores (Model II). *r*MZ, monozygotic twin correlation; *r*DZ, dizygotic twin correlation; h^2, heritability; 95% CI, 95% confidence interval.

Model	Thresholds	*r*MZ	*r*DZ	h^2 (95% CI)
I	≤23	0.79	0.46	80% (65–90%)
II	≤15, actual score	0.67	0.44	71% (61–78%)

Despite the major role of genetic factors underlying DTT scores, a certain amount of musical experience is nevertheless required to perform well on the DTT, and the original results of Kalmus and Fry provided evidence for the effects of such experience (*7*, *19*). Any effects of culture or musical experience are likely to be the same for both MZ and DZ twins and thus should have no effect on the heritability estimates that we have found. Our data indicate that individual differences in musical experience may be at least partly responsible for the fraction of variance in DTT scores attributable to unique environment (E), 20 to 29%. Because of the DTT's requirement for musical experience, it may be a conservative measure of the heritability of variation in pitch perception in isolation, that is, measured in a way that does not require such experience.

Because the FMHT serves as a rough measure of peripheral hearing, its poor correlation with the DTT suggests that musical pitch perception is largely independent of peripheral hearing and that variation in pitch perception originates in portions of the auditory system that are independent of this function.

Melodies consist of a series of tones presented in a specific order and rhythm, in which successive tones differ by specific intervals. In the DTT, the note order and rhythm remain unchanged, and only the interval between successive tones is altered. Because variation in long-term tonal memory (*9*) and musical experience appear to have modest effects, the DTT primarily determines a subject's ability to measure successive pitch intervals. Although the DTT has a number of characteristics that make it ideal for a large-scale twin study, it will be important to use other measures of pitch recognition to confirm the results obtained with the DTT. Similarly, indistinguishable distributions of DTT scores in males and females suggest that results from female subjects can be generalized to the whole population, but it will be important to confirm this with male subjects.

The original DTT was designed to identify individuals with severe deficits in pitch recognition. Our results with the modified DTT indicate a high heritability for performance across the full spectrum of pitch recognition abilities in the general population. Studies elsewhere have demonstrated a significant genetic contribution to absolute pitch (AP), a relatively rare phenomenon in which individuals are capable of identifying a particular tone without the use of a reference tone. However, AP individuals do not necessarily have superior pitch acuity compared to those without AP (*20*). In addition, the full development of AP apparently has a strong environmental component, with specific musical training (perhaps during a critical developmental time period) being required for expression (*21*). Thus, AP stands in contrast to the ability measured by the DTT, and it is not clear if the genetic factors in AP have any relation to those that underlie DTT scores.

The heritability estimates we observe for this measure of deficits in pitch recognition are very substantial and are as high as or higher than those for many common complex traits in humans (*22*). However, these high heritability estimates do not address several important issues, including the number of genes involved and their relative effects. Our results indicate that genetic approaches, which are ideal for studying traits with unknown biochemical or cellular mechanisms, are likely to be fruitful in efforts to understand this neural function.

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9. The original DTT consisted of 26 short melodies, ranging in length from 12 to 26 notes. Ten of these melodies were played normally, whereas 16 contained tonal errors (*7*). Our updated test contained 8 of the original DTT melodies verbatim, plus 18 new melodies, for a total of 26, which ranged from 12 to 26 notes in length. Of these, 9 were played correctly, whereas 17 were distorted to produce errors. The errors were introduced according to the general rules used by Kalmus and Fry in their original DTT, changing the pitch of two to nine notes, generally within one or two semitones of the correct note and following the overall rise and fall of the normal melody. As in the original DTT, all melodies were unaltered in rhythm and note order. A list of the melodies and the errors introduced is available at www.nidcd.nih.gov/intram/scientists/draynad.htm. The melodies were produced in pure tones with Mozart version 3.2 software (D. Webber, Mozart Music Software, Warrington, UK) on a Macintosh G3, rendered more natural by the addition of overtones with Arnold's MIDI (Musical Instrumentation Digital Interface) player software. Ultra Recorder version 2.4 (E. J. Campbell, EJ Enterprises, http://members.aol.com/EJC3/) was used to convert the MIDI files to AIFF (Audio Interchange File Format) and, after joining voice-over spoken instructions with SndSampler, was recorded along with spoken instructions on a

compact disk with Adaptec Toast 3.5.4. Copies of the updated DTT on a compact disk are available from the corresponding author. The updated DTT contained a preponderance of tunes played incorrectly (17/26), but our data suggest that whether a tune was played correctly or incorrectly did not significantly bias subjects' answers. Four tunes were played twice in the updated DTT; each was played correctly once and incorrectly once. Wrong answers on these eight questions were evenly distributed; 51% were correct melodies identified as incorrect, and 49% were incorrect melodies identified as correct. Two methods were used to determine the role of long-term memory and cultural experience in the performance on the updated DTT. First, subjects were asked whether they were familiar with each tune presented, and familiarity was positively correlated with correct answers. We also developed a second test, termed the International Tunes Test, that consisted of 18 short melodies chosen to be unfamiliar to all subjects and that used both Western and non-Western tonal systems. Subjects were presented with each melody played correctly twice in succession and were then asked whether a third rendition played immediately thereafter was the same as or different than the first two playings. Six of the International Tunes were played correctly the third time, whereas 12 were played incorrectly the third time. The distribution of scores on the International Test were indistinguishable from those on the updated DTT, with no significant differences between males and females. Individuals' scores on the DTT and the International Test were highly correlated (r = 0.71). These results suggest that performance on the updated DTT is not highly dependent on long-term musical memory.

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23. We thank the research nurses for skillful data collection and especially the twin volunteers who participated in this study. We also thank E. Balaban and the anonymous reviewers for their insightful comments. Supported by NIH grant Z01-DC-00043-03 from the National Institute on Deafness and Other Communication Disorders (D.D. and A.M.). M.d.L. and H.S. are sponsored by the British Heart Foundation (grants FS/99010 and FS/99050). The Twins Research Unit gratefully acknowledges support from the Arthritis and Rheumatism Council, Wellcome Trust, British Heart Foundation, and Gemini Genomics.

1 September 2000; accepted 26 January 2001

Presynaptic Kainate Receptor Mediation of Frequency Facilitation at Hippocampal Mossy Fiber Synapses

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Inhibition of transmitter release by presynaptic receptors is widespread in the central nervous system and is typically mediated via metabotropic receptors. In contrast, very little is known about facilitatory receptors, and synaptic activation of a facilitatory autoreceptor has not been established. Here we show that activation of presynaptic kainate receptors can facilitate transmitter release from hippocampal mossy fiber synapses. Synaptic activation of these presumed ionotropic kainate receptors is very fast (<10 ms) and lasts for seconds. Thus, these presynaptic kainate receptors contribute to the short-term plasticity characteristics of mossy fiber synapses, which were previously thought to be an intrinsic property of the synapse.

Neurotransmitter receptors are located on the presynaptic, as well as the postsynaptic, side of the synapse. In vertebrates these presynaptic receptors are typically metabotropic receptors and inhibit transmitter release (*1*, *2*), although in invertebrates facilitatory metabotropic actions have also been described (*3*, *4*). Ionotropic receptors are also present on presynaptic terminals (*5*), and their activation generally inhibits synaptic transmission (*6*–*11*). Although facilitation has been observed, there is no evidence that synaptically released transmitter could have access to these receptors (*5*). Kainate receptors (KARs) have recently been shown to exert presynaptic effects on glutamatergic (*10*, *12*) as well as GABAergic terminals (*9*, *13*). However, at both types of terminals activation of kainate receptors causes an inhibition of release. Here we report that activation of presynaptic kainate receptors by low levels of synaptically released glutamate enhances transmitter release at hippocampal mossy fiber synapses.

Previous studies found that application of kainate inhibits synaptic transmission at hippocampal mossy fiber synapses (*14*–*16*). However, kainate applied at concentrations considerably below those used in these previous studies strongly facilitates synaptic transmission. Kainate (50 nM) produced a robust and reversible enhancement of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) mediated excitatory postsynaptic currents (EPSCs) (Fig. 1A) (*17*), and this enhancement was associated with a decrease in paired pulse facilitation (PPF) (P2/P1: control, 2.95 ± 0.15; kainate, 1.9 ± 0.16) (Fig. 1A), suggesting that the enhancement is mediated by an increase in the probability of transmitter release (*18*). Kainate (50 nM) also produced a robust and reversible enhancement in the synaptic field potential responses (170 ± 9%), and this enhancement was also associated with a decrease in PPF (control, 2.7 ± 0.16; kainate, 1.9 ± 0.17) (*19*). To study the mechanism involved in this facilitation, AMPA and $GABA_A$ receptors were selectively blocked by GYKI 53655 and picrotoxin, respectively, and N-methyl-D-aspartate (NMDA) receptor (NMDAR)–mediated EPSCs were recorded at positive holding potentials. We used the non-NMDAR antagonists CNQX or NBQX, which block KARs, to identify effects of bath-applied kainate and synaptically released glutamate that are due to KAR activation. Low concentrations of kainate (50 nM) also reversibly enhanced NMDAR EPSCs, and this effect was completely blocked by CNQX (10 μM), indicating that the enhancement was mediated by high-affinity KARs (Fig. 1B). The enhancement of AMPAR- and NMDAR-mediated EPSCs occurred in the absence of any change in the rise time, indicating that no polysynaptic inputs were recruited (Fig. 1, A and B, insets). This same concentration of kainate also enhanced the size of the presynaptic fiber volley, and this effect was also blocked by CNQX (see Fig. 1E).

The enhancing action of kainate, both on the NMDAR EPSC (Fig. 1, A and B) and on the fiber volley, was mimicked by the addition of 4 mM K^+ to the superfusing medium (Fig. 1C), suggesting that the effect of kainate was mediated by a depolarizing action on the terminal. Neither kainate nor K^+ had any effect on the holding current, making a postsynaptic site of action most unlikely. In addition, kainate (50 nM) had no effect on

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