Cardiac Function in Mice Overexpressing the β-Adrenergic Receptor Kinase or a βARK Inhibitor

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Transgenic mice were created with cardiac-specific overexpression of the β-adrenergic receptor kinase–1 (βARK1) or a βARK inhibitor. Animals overexpressing βARK1 demonstrated attenuation of isoproterenol-stimulated left ventricular contractility in vivo, dampening of myocardial adenylyl cyclase activity, and reduced functional coupling of β-adrenergic receptors. Conversely, mice expressing the βARK inhibitor displayed enhanced cardiac contractility in vivo with or without isoproterenol. These animals demonstrate the important role of βARK in modulating in vivo myocardial function. Because increased amounts of βARK1 and diminished cardiac β-adrenergic responsiveness characterize heart failure, these animals may provide experimental models to study the role of βARK in heart disease.

β-adrenergic receptors (βARs) are the primary myocardial targets of the sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine. Human myocardium contains β_1- and β_2AR subtypes with the β_1AR being most abundant (*1*). Activation of βARs (β_1 and β_2) in the heart by agonist binding causes stimulation of adenylyl cyclase, increased intracellular concentrations of adenosine 3′,5′-monophosphate (cAMP), and increased cytosolic calcium transients resulting in positive chronotropy and inotropy (increased rate and force of contraction). As is true for most heterotrimeric guanosine triphosphate (GTP) binding protein (G protein)–coupled receptors, prolonged exposure of βARs to agonist results in a rapid decrease in responsiveness. Agonist-dependent desensitization can be initiated by phosphorylation of activated receptors by members of the G protein–coupled receptor kinase (GRK) family (*2*). The β-adrenergic receptor kinase–1 (βARK1) is a GRK that specifically phosphorylates activated β_1- (*3*) and β_2ARs (*2*) in in vitro assays. In the case of the β_1AR, these assays used cultured mammalian cells or highly purified membrane preparations containing overexpressed receptors incubated with purified recombinant βARK1 (*3*). βARK1 phosphorylation of β_1- and β_2ARs in vivo may lead to uncoupling and desensitization (*2–4*).

Chronic human heart failure is characterized by impairment of the myocardial βAR system. Human tissue samples from failing left ventricles contain 50% fewer βARs than samples from normal hearts and show decreases in β-agonist–induced adenylyl cyclase activation and inotropy (*5*). There is a selective decrease in β_1ARs in cardiac samples from patients with chronic heart failure, whereas the amount of β_2AR remains relatively constant (*5, 6*). In chronic heart failure in humans the amount of βARK1 is increased (*6*).

Cardiac overexpression of β_2ARs in transgenic mice increases myocardial contractility (*7*). Here we describe the creation of transgenic mice with cardiac-specific overexpression of βARK1 or a βARK inhibitor. The inhibitor used was a peptide corresponding to the COOH-terminus of βARK1 that competes for G protein βγ subunit binding to the enzyme, a process required for activation (*8*). This inhibitor can lead to reduced agonist-dependent GRK-mediated phosphorylation of odorant receptors (*9*) and β_2ARs (*10*). Our goal in the present work was to determine the extent to which the βARK system regulates cardiac βAR function in vivo and to assess whether this system might represent a potential therapeutic target for treatment of heart failure.

The transgene constructs consisted of the murine alpha myosin heavy chain (α-MHC) promoter ligated to either the entire coding region for bovine βARK1 or the coding sequence for only the last 194 amino acids (residues 495 to 689) of βARK1 (βARK1-minigene) (*11*). This promoter affects specific expression in adult murine atria and ventricles because α-MHC is the predominant cardiac heavy chain isoform expressed (*7, 12*). Transgenic mice were generated as described (*7, 12, 13*) with the βARK1 and βARK1-minigene transgenes. For each transgene, two lines were established (TGβK4 and TGβK12 for βARK1, and TGMini27 and TGMini33 for the βARK1-minigene) (Fig. 1). Gross phenotypic change or unusual neonatal mortality was not observed in these transgenic animals as compared with nontransgenic littermate controls. Second-generation adult animals, 2 to 4 months of age, were used for all studies.

We used protein immunoblotting to detect overexpression of βARK1 in heart extracts from TGβK12 animals and expression of the COOH-terminus of βARK1 in TGMini27 animals (Fig. 2A). To quantitate the activity of myocardial βARK in the transgenic mice, we assayed heart extracts for phosphorylation of the G protein–coupled receptor rhodopsin. Extracts from TGβK12 animals had about three times more kinase activity than that of control extracts (Fig. 2B). This enhanced activity was also observed in the presence of exogenous Gβγ and correlated with densitometry scans of protein immunoblots (*10*).

We examined the βARK inhibitory activity of myocardial extracts from TGMini27 mice. Because βARK1-minigene action is expected to result from inhibition of Gβγ activation of βARK (*8*), rhodopsin phosphorylation assays were done with exogenous Gβγ. Heart extracts (50 μg total protein) from TGMini27 mice ($n = 5$) displayed similar basal (Gβγ-independent) kinase activity to that of controls ($n = 5$) (*10*). However, upon addition of Gβγ (~10 pmol) to the assay, they showed significantly less stimulation of rhodopsin phosphorylation (2.16 ± 0.12 times that in extracts without added Gβγ) than that in controls

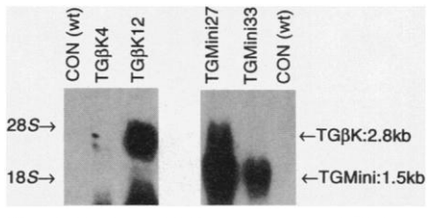

Fig. 1. Northern (RNA) analysis. Total RNA was extracted from hearts of control and transgenic mice (*23*). Total heart RNA (30 μg) was loaded on 1% agarose–formaldehyde gels, electrophoresed, transferred to nitrocellulose, and hybridized overnight at 42°C in hybridization buffer containing 50% formamide and ^{32}P-labeled SV-40 DNA as the probe. The membranes were washed twice in 0.1× saline sodium citrate at 65°C for 30 min (*7*) and subjected to autoradiography.

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(3.02 ± 0.31 times that in extracts not treated with Gβγ) ($P < 0.05$, t test). This indicates that the βARK1-minigene can compete with myocardial βARK for Gβγ binding.

To examine the biochemical effects of the two transgenes on the myocardial βAR system, we assessed receptor-effector coupling in sarcolemmal membranes from hearts of control and transgenic mice. All groups had similar amounts of receptors (~45 fmol per milligram of membrane protein). The β_1- and β_2ARs present in the mouse myocardium (*14*) differ in their ligand-binding and effector-coupling properties (*15*). To facilitate the analysis of receptor-effector coupling, we focused on the predominant β_1 subtype (*14*) and examined competition binding isotherms with the β-agonist isoproterenol. Control isotherms (Fig. 3A) were biphasic and could be modeled to a high-affinity (0.62 nM) and low-affinity (32 nM) component. A portion (44%) of the receptors were in the high-affinity state that represents a ternary complex of hormone (H), receptor (R), and G protein (G) (*16*). GTP shifted all receptors to the low-affinity state. Isotherms from TGβK12 cardiac membranes were shifted to the right of those for the control membranes (Fig. 3A). GTP caused both curves to shift to the right such that the curves were superimposable (Fig. 3A). Computer modeling indicated that the changes found in the binding isotherms resulted from a decreased ability of the β_1ARs from the TGβK12 animals to form the coupled H-R-G high-affinity state [28% for receptors in TGβK12 animals compared with 44% for receptors from littermate control animals ($P < 0.05$, F test)] with no change in affinity for the receptors. Such changes occur when receptors are desensitized (*17*). In contrast, receptor-binding isotherms of TGMini27 mice did not differ from those of control mice (*10*).

In cardiac membranes from TGMini27 mice, the adenylyl cyclase activity was stimulated by β_1ARs to the same extent as in control membranes (*10*). In contrast, TGβK12 mice had significantly lower basal and agonist-stimulated cyclase activities (Fig. 3B), probably because of increased phosphorylation of β_1ARs by the overexpressed βARK in response to normal cardiac sympathetic tone and basal circulating amounts of epinephrine. Thus, functional coupling of β_1AR, as assessed by ligand binding and adenylyl cyclase activation, is hindered by the overexpression of βARK1. The reason for the absence of any difference in β_1AR binding or cyclase activation in sarcolemmal membranes from TGMini27 hearts may be that in contrast to βARK, some of which associates with membranes, the βARK1-minigene peptide is a soluble protein that would not be expected to be present in sarcolemmal membranes.

To investigate the effect of βARK1 and the βARK1-minigene on in vivo myocardial function, we used cardiac catheterization in anesthetized intact mice (*7*, *18*). Continuous measurements of heart rate (HR), left ventricular (LV) pressures, and aortic pressure were recorded at baseline and after progressive doses of isoproterenol. For TGβK12 mice at baseline, HR, the maximum and minimum first derivative of the LV pressure (LV dP/dt_{max} and LV dP/dt_{min}), and LV systolic pressure were similar to those in control animals (Fig. 4). However, the inotropic and chronotropic responses to isoproterenol in TGβK12 animals were significantly blunted (Fig. 4, A to C). The isoproterenol-induced rise in LV systolic pressure was also attenuated in the TGβK12 animals as compared with control mice but did not reach statistical significance (Fig. 4D). Thus, the in vivo effects of overexpressed βARK1 mimic those seen in vitro and suggest that cardiac β_1- and β_2ARs are targets for βARK1-induced desensitization. The enhanced desensitization of βARs in TGβK12 animals suggests that βARK1 may function in the regulation of myocardial contractility and supports the hypothesis that the diminished catecholamine responsiveness seen in heart failure may in part result from increased expression of βARK1 (6).

At baseline, LV dP/dt_{max} (Fig. 5A), LV dP/dt_{min} (Fig. 5B), and LV systolic pressure (Fig. 5D) for TGMini27 mice were all statistically increased compared with values from control animals. In TGMini27 animals, LV responses to isoproterenol were also enhanced (Fig. 5, A, B, and D). The basal and the isoproterenol-stimulated HR in TGMini27 mice were similar to that in controls (Fig. 5C). A second line of mice (TGMini33) expressing the βARK1-minigene (Fig. 1) had essentially the same phys-

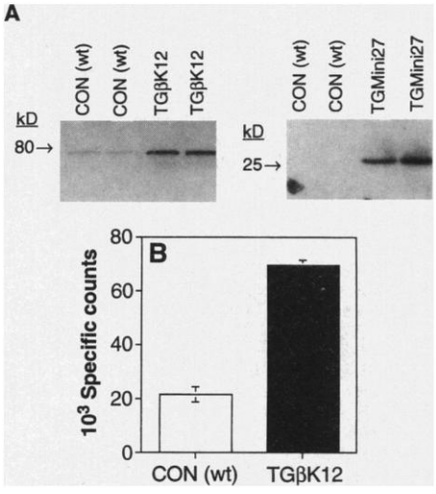

Fig. 2. Assessment of protein expression. (**A**) Protein immunoblot analysis of βARK1 and the βARK1-minigene in transgenic mice. Extracts from control or transgenic hearts were prepared (*22*) and equal amounts of protein (20 to 40 μg) were electrophoresed through 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Transgene products were identified with rabbit polyclonal antiserum to the COOH-terminus of βARK1 (*8*) and by chemiluminescent detection of alkaline phosphatase–conjugated secondary antibodies. Molecular sizes are indicated in kilodaltons. (**B**) Assessment of GRK activity in myocardial extracts from TGβK12 mice. The data shown represent means ± SE of heart extracts ($n = 4$) prepared (*22*) and used in a rhodopsin phosphorylation assay (*24*). Extracts from TGβK4 mice, which had less abundant mRNA expression (Fig. 1), catalyzed only ~50% more rhodopsin phosphorylation than control extracts (*10*).

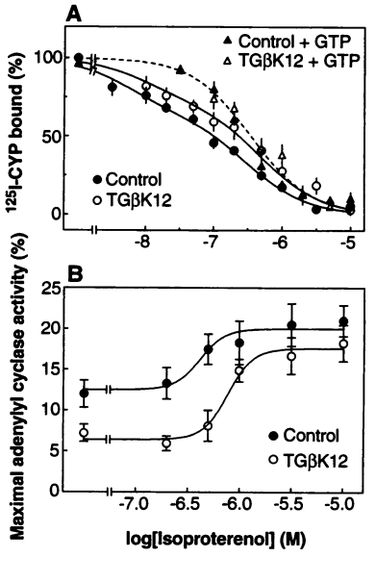

Fig. 3. Effect of βARK1 transgene expression on β_1AR receptor-effector coupling. (**A**) Isoproterenol competition binding isotherms with sarcolemmal membranes (*20*) prepared from the hearts of TGβK12 and nontransgenic littermate controls. Isotherms (*21*) on the β_1AR (*14*) were done in the absence or presence of GTP (100 μM). Data represent the means ± SE ($n = 3$ to 9 hearts). The isotherms (*21*) in the absence of GTP are statistically different ($P < 0.05$, F test). (**B**) Adenylyl cyclase activity due to β_1AR stimulation in sarcolemmal membranes (*20*) of TGβK12 and control hearts ($n = 3$) was determined under basal conditions and in the presence of various doses of isoproterenol or 10 mM NaF (*7*). Membranes (5 to 20 μg of protein) were incubated for 10 min at 37°C, and [α-^{32}P]ATP was isolated and cAMP quantitated (*25*). Basal and isoproterenol-stimulated cyclase values were normalized to the percentage of activation achieved with NaF, which was not significantly different between transgenic and control membranes (195 ± 33 pmol/mg per minute in membranes from TGβK12 animals compared with 139 ± 22 pmol/mg per minute in membranes from control animals; P, not significant, t test). Data shown represent the means ± SE. Basal values of TGβK12 membranes as well as the entire isoproterenol dose-response curve were statistically significant compared with values from control membranes ($P < 0.05$, F test).

iological phenotype (*10*). The enhanced myocardial function in response to expression of the βARK inhibitor, which is the opposite cardiac phenotype of that seen in TGβK12 mice, approached that observed in transgenic mice with cardiac-specific overexpression of β_2AR (*7*).

The observed alterations of βAR function and LV contractility were unlikely to be due to differences in loading conditions, because LV end diastolic pressure (EDP) was essentially identical in all groups (under basal conditions, LV EDP for TGβK12 animals was 3.99 ± 0.31 mmHg compared with 3.97 ± 0.71 mmHg for the control animals; basal LV EDP for TGMini27 animals was 4.06 ± 0.47 mmHg compared with 4.87 ± 1.09 mmHg in control animals). Acute changes in LV systolic pressure did not seem to alter LV contractility in the intact mouse, as documented by separate experiments with control animals that showed no difference in LV dP/dt_{max} during transient aortic constriction (*19*). The reciprocal nature of the physiologic phenotypes of TGβK12 and TGMini27 mice (Figs. 4 and 5) suggests that the βARK1-minigene is acting through βARK inhibition and that βARK1 is a critical in vivo modulator of myocardial function (mediated by βARs) both under baseline conditions and after sympathetic stimulation.

It is unclear why basal cardiac function was altered only in the presence of the βARK inhibitor. Under basal conditions in vivo, the concentration of βARK may not be rate-limiting, but rather the availability of Gβγ (that is, agonist-occupied receptor) may determine the level of receptor phosphorylation and thus of βAR uncoupling. Our in vivo data support this hypothesis because overexpressed βARK has little physiological effect under basal conditions.

Our findings support the concept that increased myocardial βARK1 may be a pathological element in chronic heart failure, because increased βARK1 activity in normal hearts blunted the inotropic response to isoproterenol. This effect appears to result from a decrease of receptor coupling to G protein, which is reflected in impaired formation of the high-affinity state of β_1ARs. These data together with in vitro data (*3*) suggest that β_1AR is a substrate for βARK action. The phenotype of TGMini27 mice demonstrates a mechanism for the induction of positive inotropy—the reduction of βAR desensitization by βARK inhibition.

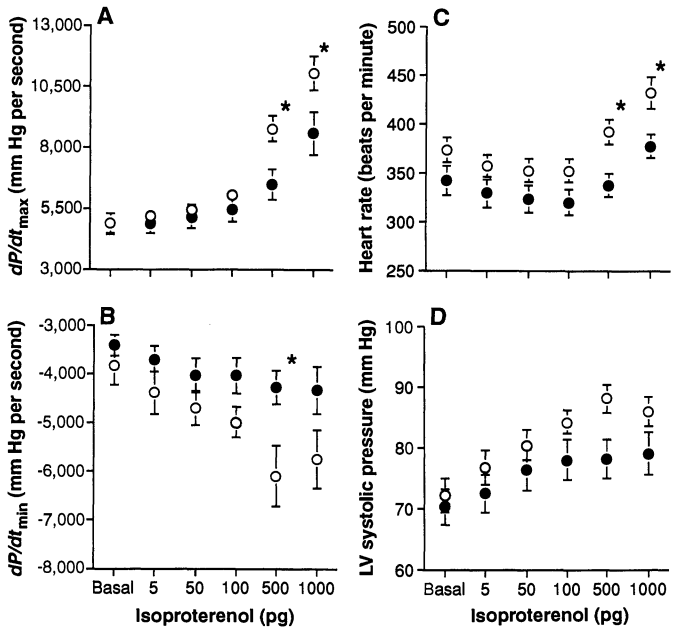

Fig. 4. In vivo assessment of left ventricular (LV) function of TGβK12 (n = 10) (●) and nontransgenic control mice (n = 10) (○). Animals were anesthetized and aortic and LV catheters placed (*26*). Four measured parameters are shown at baseline and after progressive doses of isoproterenol: (**A**) LV dP/dt_{max}; (**B**) LV dP/dt_{min}; (**C**) heart rate (HR); and (**D**) LV systolic pressure. Data are reported as means ± SE and were analyzed with a two-way repeated measures analysis of variance (ANOVA). Post hoc testing was done with Scheffe's F test (*, $P < 0.05$). When the entirety of the control and TGβK12 isoproterenol dose response curves were compared by ANOVA, the pattern of change for the groups was found to differ significantly for (A) dP/dt_{max}, $P < 0.001$; (B) dP/dt_{min}, $P < 0.05$; and (C) HR, $P < 0.01$, but not for (D) LV systolic pressure.

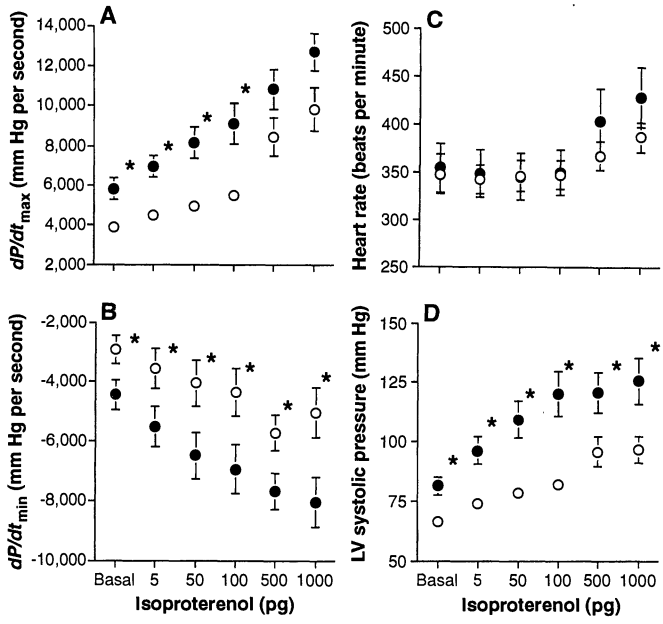

Fig. 5. In vivo assessment of LV function of TGMini27 (n = 7) (●) and nontransgenic control mice (n = 7) (○). Mice were treated as in Fig. 4 and four parameters are shown: (**A**) LV dP/dt_{max}; (**B**) LV dP/dt_{min}; (**C**) HR; and (**D**) LV systolic pressure. Data are reported as means ± SE and analyzed as in Fig. 4 (*, $P < 0.05$). A significant between-group main effect in response to isoproterenol was found for (A) dP/dt_{max}, $P < 0.005$; (B) dP/dt_{min}, $P < 0.005$; and (D) LV systolic pressure, $P < 0.005$. The pattern of change between groups was statistically different only for (D) LV systolic pressure, $P < 0.05$.

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11. For construction of the βARK1 transgene, a 5.5-kb Sac I–Hind III fragment, the α-MHC promoter, was isolated [A. Subramanian *et al.*, *J. Biol. Chem.* **266**, 24613 (1991)] and ligated to the 2.1-kb Hind III–Bam HI fragment of bovine βARK1 engineered to contain only the coding region [J. Inglese, W. J. Koch, M. G. Caron, R. J. Lefkowitz, *Nature* **359**, 149 (1992)] and inserted as a Sac I–Bam HI fragment into a plasmid containing the SV-40 intron–polyadenylate signal as described (*7*) to generate pGEM–α-MHC–βARK1–SV-40. The βARK1-minigene DNA was as described [W. J. Koch, B. E. Hawes, J. Inglese, L. M. Luttrell, R. J. Lefkowitz, *J. Biol. Chem.* **269**, 6193 (1994)] except that new 5′ and 3′ restriction enzyme sites were inserted by the polymerase chain reaction to generate a 0.8-kb Sal I–Hind III fragment. This fragment was ligated with the 5.5-kb Sac I–Sal I α-MHC promoter fragment into pGEM SV-40 generating pGEM-

α–MHC–βARK1–minigene–SV-40. The transgenes were linearized and purified before microinjection.
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13. Institutional review board approval for all mouse experiments was obtained at Duke University, the University of Houston, and the University of California at San Diego.
14. Sarcolemmal membranes (*20*) were prepared from control mouse hearts, and competition binding isotherms were determined for the β_2-selective ligand ICI 118,551 [erythro-dl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol] against the nonselective ligand ^{125}I-cyanopindolol (^{125}I-CYP). Two binding populations were thus identified: 25% high affinity [K_i (inhibition constant) = 0.9 nM; β_2 subtype] and 75% low affinity (K_i = 390 nM; β_1 subtype). Subsequent isoproterenol-binding isotherms (*21*) contained 75 nM ICI 118,551 in order to selectively study β_1AR binding.
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20. Myocardial sarcolemmal membranes were prepared by homogenization of whole hearts in ice-cold buffer A [50 mM Hepes (pH 7.3), 150 mM KCl, and 5 mM EDTA]. Nuclei and tissue were separated by centrifugation at 800*g* for 10 min and the crude supernatant was then centrifuged at 20,000*g* for 10 min. Sedimented proteins were resuspended at a concentration of 2 to 3 mg protein per milliliter in buffer B [50 mM Hepes (pH 7.3), and 5 mM $MgCl_2$].
21. Competition binding isotherms in sarcolemmal membranes (*20*) were done with 80 pM ^{125}I-CYP and varying amounts of isoproterenol in 250 μl of binding buffer [50 mM Hepes (pH 7.3), 5 mM $MgCl_2$, 0.1 mM ascorbic acid, and 75 nM ICI 118,551] with or without 100 μM GTP. Assays were done at 37°C for 1 hour and then filtered over glass fiber filters, which were washed twice and counted in a gamma counter. Data were analyzed by nonlinear least-square curve fitting [P. J. Munson and D. Rodbard, *Anal. Biochem.* **107**, 220 (1980)]. The data in Fig. 3A could not be fitted to the same parameters and represent the best simultaneous fit of the data with shared high- and low-affinity values.
22. We prepared myocardial extracts essentially as described (*6*) by homogenization of whole hearts in ice-cold lysis buffer (2 ml) [25 mM tris-HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, leupeptin (10 μg/ml), aprotinin (20 μg/ml), and 1 mM phenylmethylsulfonyl fluoride]. The crude homogenate was centrifuged for 30 min at 20,000*g*. Sedimented material was discarded and protein concentrations of the supernatants were determined [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. TGβK heart samples were further purified by addition of NaCl (to a final concentration of 50 mM) and 0.75 ml of a slurry of 50% (v/v) diethylaminoethyl Sephacel (pH 7.0). This mixture was incubated on ice for 30 min and poured over a small disposable column. Final supernatants were eluted with 1 ml of cold lysis buffer and concentrated with a Centricon (Amicon) microconcentrator. βARK1-minigene expression was assessed directly from concentrated supernatants.
23. Total RNA was extracted with RNAzol (Biotecx Laboratories, Houston, TX) [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)].
24. GRK activity of heart extracts was determined in concentrated supernatants from TGβK and control mice (*22*). Extracts (50 μg protein) were incubated with rhodopsin-enriched rod outer segments (*8*) in lysis buffer (75 μl) (*22*) with 10 mM $MgCl_2$ and 0.1 mM adenosine triphosphate (ATP) (containing [γ-^{32}P]ATP). After incubation in white light for 15 min at room temperature, reactions were quenched with ice-cold lysis buffer (300 μl) and centrifuged for 15 min at 13,000*g*. The sedimented proteins were resuspended in 20 μl of lysis buffer and electrophoresed on 12% SDS-polyacrylamide gels. The gels were dried and phosphorylated rhodopsin was quantitated with a PhosphorImager (Molecular Dynamics).
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26. Mice were anesthetized with a mixture of ketamine and xylazine (*7*). After endotracheal intubation, mice were connected to a volume-cycled ventilator. Either carotid artery was cannulated with a flame-stretched PE 50 catheter connected to a modified P50 statham transducer. The chest was then opened and a 2F high-fidelity micromanometer catheter was inserted into the left atrium, advanced through the mitral valve, and secured in the LV. Hemodynamic measurements were recorded at baseline and 45 to 60 s after injection of incremental doses of isoproterenol. Continuous pressures were recorded on an eight-channel chart recorder and in digitized form on computer disk for beat averaging. Ten sequential beats were averaged for each measurement.
27. We thank J. Robbins for the murine α-MHC promoter; C. Bock, W. C. Stone, and N. Rahman for technical assistance; M. Tiberi for assistance in computer analysis; N. Freedman and P. Dolber for helpful discussions during the course of this work; and D. Addison and M. Holben for secretarial assistance. Supported in part by NIH grants HL-16037 (R.J.L.) and 5F32-CA09350 (C.A.M.) and by the American Heart Association, California affiliate grant 94G-286 (H.A.R.) and Texas affiliate grant 94G-458 (R.A.B.).

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Sensorimotor Encoding by Synchronous Neural Ensemble Activity at Multiple Levels of the Somatosensory System

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Neural ensemble processing of sensorimotor information during behavior was investigated by simultaneously recording up to 48 single neurons at multiple relays of the rat trigeminal somatosensory system. Cortical, thalamic, and brainstem neurons exhibited widespread 7- to 12-hertz synchronous oscillations, which began during attentive immobility and reliably predicted the imminent onset of rhythmic whisker twitching. Each oscillatory cycle began as a traveling wave of neural activity in the cortex that then spread to the thalamus. Just before the onset of rhythmic whisker twitching, the oscillations spread to the spinal trigeminal brainstem complex. Thereafter, the oscillations at all levels were synchronous with whisker protraction. Neural structures manifesting these rhythms also exhibited distributed spatiotemporal patterns of neuronal ensemble activity in response to tactile stimulation. Thus, multilevel synchronous activity in this system may encode not only sensory information but also the onset and temporal domain of tactile exploratory movements.

The processing of somatosensory information in the mammalian brain involves the transmission of neural activity from the skin to the neocortex by way of parallel pathways that ascend through a hierarchical sequence of neural structures (*1*). Like those in other sensory systems, these ascending pathways are far outnumbered by corticofugal descending projections (*2*), which can act at multiple subcortical levels to modify the processing of sensory information (*3*). These connections define a recurrent network that is theoretically capable of generating complex emergent dynamic patterns of neural activity, manifested by synchronous oscillations or even chaotic behavior (*4*), that could be computationally useful (*5*). The existence of this network raises the question of whether large-scale coordinated activity of neural ensembles involving multiple levels of a sensory system (that is, brainstem, thalamus, and cortex) can play a fundamental role in the coding of sensory information. To address this issue, we simultaneously recorded up to 48 single neurons, distributed across up to five distinct processing relays of the trigeminal somatosensory system, in freely behaving rats (*6*). The rat trigeminal system is a multilevel, recurrently interconnected neuronal network, specialized for processing complex patterns of tactile stimuli generated by the repetitive contacts of facial whiskers with surrounding objects. Rats rely on rhythmic movements of their facial whiskers, much as humans rely on coordinated movements of their fingertips, to discriminate object shape and texture (*7*). Although the properties of single neurons belonging to this sensory system have been well studied under anesthetized con-

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