Evidence of a role for cyclic ADP-ribose in long-term synaptic depression in hippocampus

(CA1 region/cyclic GMP/ryanodine/synaptic plasticity)

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Communicated by Michael V. L. Bennett, Albert Einstein College of Medicine, Bronx, NY, December 31, 1998 (received for review September 23, 1998)

ABSTRACT Ca2+ released from presynaptic and postsynaptic intracellular stores plays important roles in activity-dependent synaptic plasticity, including long-term depression (LTD) of synaptic strength. At Schaffer collateral–CA1 synapses in the hippocampus, presynaptic ryanodine receptor-gated stores appear to mobilize some of the Ca2+ necessary to induce LTD. Cyclic ADP-ribose (cADPR) has recently been proposed as an endogenous activator of ryanodine receptors in sea urchin eggs and several mammalian cell types. Here, we provide evidence that cADPR-mediated signaling pathways play a key role in inducing LTD. We show that biochemical production of cGMP increases cADPR concentration in hippocampal slices in vitro, and that blockade of cGMP-dependent protein kinase, cADPR receptors, or ryanodine-sensitive Ca2+ stores each prevent the induction of LTD at Schaffer collateral–CA1 synapses. A lack of effect of postsynaptic infusion of either cADPR antagonist indicates a probable presynaptic site of action.

Recently discovered messenger molecule, cyclic ADP-ribose (cADPR), offers an intriguing possible connection. In sea urchin eggs (3) and mammalian cells (4, 5), cGMP has been shown to stimulate a cyclase/hydrolase that synthesizes cADPR. There is a cADPR binding site on ryanodine-sensitive channels which, when stimulated, enhances Ca2+-triggered release of Ca2+ from this intracellular pool. We tested the hypothesis that cADPR might be a crucial messenger mediating the induction of LTD, by biochemically measuring cADPR synthesis and testing the ability of two selective membrane-permeant cADPR antagonists to impair induction of LTD at Schaffer collateral–CA1 synapses in hippocampal slices in vitro.

MATERIALS AND METHODS

Electrophysiological Recordings from Hippocampal Slices. Transverse hippocampal slices were prepared from 14- to 21-day-old Sprague–Dawley rats of either sex sacrificed while they were under deep ether anesthesia. The hippocampus plus entorhinal cortex was dissected out, and 400-μm-thick slices were cut simultaneously with a spring-loaded mechanism ("egg slicer") that rapidly forces a parallel grid of 20-μm-diameter wires through the tissue. Slices recovered for 1 hr at 33°C in a humidified, oxygenated (95% O2/5% CO2) interface recording chamber continuously recirculating 2 ml of artificial cerebrospinal fluid (ACSF). ACSF composition (in mM) was NaCl 126, NaHCO3 26, NaH2PO4 1.25, KCl 5, CaCl2 2, MgCl2 2, d-glucose 10, pH 7.4.

Two separate Schaffer collateral-commisural axon populations were isolated by placing stimulating electrodes in the stratum radiatum on opposite sides of the recording site, verified as separate inputs by a lack of paired-pulse facilitation (50-ms interval), and alternately stimulated each 30 s with bipolar stainless steel electrodes (Frederick-Haar; 150-μs dc square pulses). Extracellular recording electrodes (Ri = 2–5 MΩ) were filled with 2 M NaCl. We adjusted stimulus intensity so population excitatory postsynaptic potential (EPSP) amplitude was 50% of maximum (＞2 mV), as determined by input/output curves.

LTD was evoked by a low-frequency stimulus (LFS) train (150-μs pulses at 2 Hz for 10 min). Maximum initial slopes of field and intracellular EPSPs were calculated by using a six-point interpolation least-squares linear regression method.

Abbreviations: cADPR, cyclic adenosine 5′-diphosphate-ribose; EPSP, excitatory postsynaptic potential; LFS, low-frequency stimuli; LTD, long-term depression; LTP, long-term potentiation; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; Rp-sPcPT-cGMPS, Rp-8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphorothioate; RyR, ryanodine receptor.

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Each point is from slopes normalized to pre-LFS baselines, averaged over all experiments ± SEM. Changes in synaptic strength were assessed as percent change between baseline averages 10 min preceding LFS and the average of 10 points spanning the 30-min post-LFS period. Statistical significance was determined by a two-tailed Student’s t test with significance level P < 0.05. Drugs were divided into aliquots and frozen in 1000× stock solutions in distilled H2O (extracellular), 2 M potassium acetate (intracellular), or DMSO in the case of ryanodine (Research Biochemicals), and they were diluted immediately before use. 7-Deaza-8-Br-cADPR and 8-NH2-cADPR were synthesized as described (6), purified on a column of Q-Sepharose Fast Flow (Sigma) using a gradient of triethylammonium bicarbonate as buffer, isolated as triethylammonium salts, and fully characterized by UV, NMR, and mass spectrometry.

Intracellular evoked EPSPs were recorded from CA1 pyramidal neurons (resting membrane potential = −63.6 ± 0.5 mV; Rm = 49 ± 2.5 MΩ; n = 15) impaled with sharp microelectrodes (80–120 MΩ), 2 M potassium acetate. Sharp electrodes were used to prevent rundown of cells caused by washout of intracellular factors. To facilitate infusion of cADPR antagonists into pyramidal neurons, biphasic current pulses of 0.2–0.5 nA magnitude, 200-ms duration were injected at a frequency of 1 Hz for 10–15 min prior to the start of recording baseline EPSPs. In all experiments, simultaneous field recordings were made to monitor spike stability.

Biochemical Measurement of ADP-Ribosyl Cyclase Activity. ADP-ribosyl cyclase activity was measured in hippocampal homogenates by bioassay of activity of product on sea urchin egg homogenate. Hippocampi from five rats were gently homogenized in 0.25 M sucrose/10 mM Hepes buffer, pH 7.2, and centrifuged at 1,000 × g at 4°C. To test for cyclase activity, supernatant was incubated with 2.5 mM β-NAD+ at 37°C for 10–90 min, and ADP-ribosyl cyclase activity was determined by adding 5 µl of incubation supernatant to sea urchin egg homogenate and measuring evoked increase in Ca2+ concentration with Fluo-3 (Molecular Probes). Fluorescence was calibrated daily against signals generated by known concentrations of synthetic cADPR (10–50 nM). All measurements were done in triplicate, on 6 separate days with material from 2–4 rats. In parallel experiments (n = 6), hippocampal homogenates degraded labeled cADPR (75% ± 15% reduction in cADPR concentration after 90 min), consistent with the bifunctional nature of ADP-ribosyl cyclase/hydrolase.

Biochemical Measurement of cADPR Concentration. cADPR concentration was measured in hippocampal slices by a method similar to radioimmunoassay, replacing antibody with purified cADPR receptor from sea urchin egg homogenate. Hippocampal slices (12–14) were prepared by using a Vibroslicer (Camden Instruments) and placed in an interface chamber at room temperature (25°C). After 1-hr preincubation in normal ACSF, slices were treated for 30 min with either zaprinast (20 µM) or zaprinast plus inhibitor H89 (10 µM) to prevent coactivation of cAMP-dependent protein kinase (PKA). After treatment, slices were immersed in ice-cold Ca2+-free ACSF for three volume exchange washes to block synaptic transmission and reduce metabolism, and they were stored at −80°C. Samples were thawed, excess ACSF was removed, and the samples were disrupted vigorously in 200 µl of glucuronate-based Ca2+-free buffer. Aliquots were taken from all samples for protein determination by standard methods.

A standard binding curve was generated with 10 nM [3H]cADPR plus various concentrations of unlabeled cADPR (1 nM to 10 µM), in 250 µl of glucuronate buffer, plus 10% sea urchin egg homogenate (0.3–0.4 mg/ml). After 25-min incubation on ice, binding was interrupted by filtration over GF/C filters (Sigma) and three washes with 5 ml of ice-cold buffer. Filters were collected and radioactivity was measured in a scintillation counter for 1 min. A standard curve was plotted for cADPR concentration versus radioactivity minus nonspecific binding.

To extract cADPR, 50 µl of slice homogenate was treated with 50 µl of 1 M trichloroacetic acid to precipitate all proteins, and was centrifuged at 1,000 × g for 2 min. Supernatant was neutralized with 25 µl of 1 M Na2CO3, and 5- or 10-µl aliquots were added to the radioassay in place of unlabeled cADPR. On the linear portion of the standard curve, cADPR concentration in the deproteinized sample was calculated and corrected to pmol/mg of protein in the original sample.

**RESULTS**

Fig. 1A illustrates normal LTD of synaptic strength at Schaffer collateral-CA1 synapses in hippocampal slices in vitro. When a prolonged train of LFS (2 Hz/10 min; solid bar) was applied to Schaffer collateral axons, a reduction in the initial slope of evoked EPSPs was elicited. After LFS, stable LTD was observed which persisted for at least 1 hr (−41% ± 8% decrease from pre-LFS baseline EPSP slope) and was confined to the stimulated set of synapses (control input not shown). Because some of the antagonists used for these studies were in limiting supply, all experiments (including controls) where drugs were bath applied were conducted in a recirculating chamber with a total volume of ∼2 ml.

**Release of Ca2+ from Ryano...**

**Inhibitors of PKG Block Induction of LTD.** Evidence suggests that a major component of LTD depends on activation of presynaptic guanylyl cyclase by the retrograde messenger NO (2, 7). In other cells, the second messenger cGMP has been shown to elevate intracellular Ca2+ concentration by stimulating production of cADPR by ADP-ribosyl cyclases (8, 9), a process thought to be dependent on activation of PKG. To examine directly the idea that such a cascade plays a role in the induction of LTD, we tested the necessity for PKG activity. Fig. 1C illustrates these experiments, in which the cell-permeant, hydrolysis-resistant PKG inhibitor Rp-8pCPT-cGMPS (Bio-Mol, Plymouth Meeting, PA) (10) was bath applied (10 µM; hatched bar) 30 min before LFS (2 Hz/10 min; solid bar). As with ryanodine, inhibition of PKG blocked the induction of LTD at Schaffer collateral synapses, consistent with a presynaptic site of action.

**Raising cGMP Concentration in Hippocampal Slices Stimulates Synthesis of cADPR.** Given that cGMP, PKG, and RyR-gated calcium stores are all necessary for the induction of LTD, it seemed a reasonable hypothesis that cADPR could be the messenger linking cGMP to release from calcium stores. If this hypothesis is true, the hippocampus should be able to synthesize cADPR, and raising cGMP concentration should...
stimulate cADPR production. Fig. 2A illustrates synthesis of cADPR in hippocampal homogenates. To test for ADP-ribosyl cyclase activity, rat hippocampi were homogenized and the 1,000 g supernatant was incubated with $\beta$-NAD for periods ranging from 10 to 90 min. cADPR concentration was then bioassayed by application to sea urchin egg homogenate, and Ca$^{2+}$ released from intracellular stores was measured by

![Figure 2A](image1)

**FIG. 2.** Hippocampal slices possess cGMP-stimulated ADP-ribosyl cyclase activity. (A) (Upper) Fluoro-3 fluorescence (relative fluorescence units; rfu) increases in sea urchin egg homogenate produced by known concentrations of cADPR (arrow). Numbers next to each trace are cADPR in nmol, against which hippocampal cADPR activity was calibrated. (Lower) Time course of cADPR synthesis in hippocampal homogenates incubated with 2.5 mM $\beta$-NAD for 30 min. After the indicated times, reaction was stopped and 5 ml of incubation medium was added to sea urchin homogenate (n = 6) for fluorescence measurement of amount of cADPR. (B) Mean ± SEM amount of cADPR in control hippocampal slices (open bars), versus slices treated with 20 $\mu$M zaprinast (ZAP; hatched bar), a type V phosphodiesterase inhibitor that raises cGMP concentration by preventing its degradation, and 20 $\mu$M zaprinast plus the PKA inhibitor H89 (10 $\mu$M; solid bar). n = 12–14 slices per group in three experiments. * P < 0.05, Student’s t test compared with control slices.

![Figure 1](image2)

**FIG. 1.** Blockade of induction of LTD by ryanodine and the cGMP-dependent protein kinase (PKG) inhibitor Rp-8-(4-chlorophenylthio) guanosine 3',5'-cyclic monophosphorothioate (Rp-8pCPT-cGMPS). The first derivative of the EPSP (fEPSP, EPSP slope) is plotted as a function of time. (Insets) Single extracellular fEPSPs recorded at times indicated. Scales indicate 2 mV and 4 ms. (A) Control LTD of synaptic strength at Schaffer collateral–CA1 synapses in hippocampal slices (n = 5). Slices were placed in an interface recording chamber continuously recirculating 2 ml of ACSF. After stable baseline EPSPs had been recorded for at least 30 min, LFS (2 Hz/10 min; solid bar) evoked LTD that remained stable throughout the 1-hr recording period after LFS. (B) When 10 $\mu$M ryanodine (hatched bar) was bath applied (n = 4) 30 min before LFS (solid bar), the magnitude of LTD evoked was markedly and significantly reduced (P < 0.05, Student’s t test compared with control LTD). (C) When 10 $\mu$M Rp-8pCPT-cGMPS (hatched bar) was bath applied (n = 8) 30 min before LFS (solid bar), LTD was also markedly reduced in amplitude (P < 0.05, Student’s t test compared with control LTD).
Fluo-3 fluorescence and compared with fluorescence induced in the same homogenate by known concentrations of synthetic cADPR. Fig. 2A shows the time course of cADPR synthesis, while Upper shows fluorescence signals calibrated for concentration. Previous work confirmed that antagonists of cADPR binding sites prevent the increases in intracellular Ca\(^{2+}\) concentration evoked in this system (6, 12).

Fig. 2B summarizes direct measurement of cADPR production stimulated by raising cGMP concentration in hippocampal slices. To raise cGMP concentration, we applied the phosphodiesterase inhibitor zaprinast, which selectively inhibits cGMP-selective (type V) phosphodiesterase (13). Zaprinast, when applied alone at 20 \(\mu M\) to hippocampal slices for 30 min (hatched bar), did not significantly change cADPR produced compared with control slices (open bars). However, when zaprinast (20 \(\mu M\)) was applied along with the PKA inhibitor H89 (10 \(\mu M\)) to block PKA activation that might counteract cGMP, it did elicit a sustained increase in cADPR concentration (151% \(\pm\) 16%; \(P < 0.05\), Student’s \(t\) test compared with untreated slices). The rationale

**FIG. 3.** Blockade of LTD by cell-permeant cADPR receptor antagonists. (A) Time course \((n = 5)\) of LTD induced by LFS (2 Hz/10 min; solid bar) in the presence of 100 \(\mu M\) 8-Br-cADPR (hatched bar). At this concentration, 8-Br-cADPR had no effect on the magnitude or duration of LTD. (B) A 5-fold higher concentration of 8-Br-cADPR (500 \(\mu M\); hatched bar) produced a partial reduction in the magnitude of LTD \((P < 0.05, \text{Student’s } t \text{ test compared with control LTD}; n = 4)\). (C) A more potent, nonhydrolyzable cADPR antagonist, 7-deaza-8-Br-cADPR, produced an equivalent partial blockade at a 50 \(\mu M\) concentration (hatched bar; \(n = 5\)). (D) At 100 \(\mu M\), 7-deaza-8-Br-cADPR (hatched bar) completely blocked the induction of LTD by LFS (solid bar; \(P < 0.05, \text{Student’s } t \text{ test compared with Control LTD}, 500 \(\mu M\) 8-Br-cADPR, and 50 \(\mu M\) 7-deaza-8-Br-cADPR; \(n = 4\)). (E) In contrast to LTD, 100 \(\mu M\) 7-deaza-8-Br-cADPR (hatched bar) did not impair the induction of LTP by high-frequency stimulation (TET-ANUS; 100 Hz/500 ms in six trains; \(n = 4\)).

**FIG. 4.** Postsynaptic intracellular injection of cADPR antagonists into CA1 pyramidal neurons does not affect the induction of LTD. (A) Time course of LTD of Schaffer collateral-evoked intracellular EPSPs in CA1 pyramidal neurons \((n = 4)\) impaled with microelectrodes containing 1 mM 8-Br-cADPR. Before baseline recording was commenced, 10–15 min of depolarizing current pulse injection \((0.2–0.5 \text{nA/200 ms at 1 Hz})\) was applied to facilitate drug infusion. In contrast to extracellular application, postsynaptic 8-Br-cADPR did not alter the magnitude or duration of LTD. As in controls, LTD was homosynaptic (collateral input not shown). (B) Similarly, postsynaptic intracellular infusion of 1 mM 7-deaza-8-Br-cADPR \((n = 7)\) was also unable to block or impair expression of LTD at Schaffer collateral–CA1 synapses. (Insets) Single intracellular EPSPs recorded at times indicated. Scale bars represent 5 mV and 10 ms.
for this co-application protocol arose from our recent discovery that elevating cGMP concentration elicits LTD in field CA1 only when combined with PKA inhibition (14).

Antagonists of cADPR-Stimulated Ca\(^{2+}\) Release Block Induction of LTD, but Not LTP. To test the hypothesis that a cADPR binding site is a necessary factor in the induction of LTD, we utilized two different membrane-permeant cADPR antagonists; 8-Br-cADPR (6, 12), and a more potent analog, 7-deaza-8-Br-cADPR, which is a much poorer substrate for cADPR hydrolase (6). Fig. 3 illustrates the results of these experiments, which were also performed in the recirculating chamber. In Fig. 3A, 100 μM 8-Br-cADPR (hatched bar) was present in the bath for at least 60 min before the start of the experiment. After 30 min of baseline recording of extracellular EPSPs, LFS (2 Hz/10 min; solid bar) was applied to Schaffer collateral axons, and normal LTD was elicited (241% ± 6%). In contrast, when a separate group of slices (Fig. 3B) was pretreated with 500 μM 8-Br-cADPR, LTD was markedly reduced, but not completely blocked (217% ± 7%; \(P < 0.05\), Student’s \(t\) test compared with control LTD). At a higher concentration (100 μM), the induction of LTD was blocked completely (Fig. 3D; +7% ± 8%, \(P < 0.05\), Student’s \(t\) test compared with control LTD). In contrast, the same high concentration of 7-deaza-8-Br-cADPR in a separate group of slices (\(n = 4\)) did not impair LTP induced by high-frequency stimuli (six trains of 100 Hz/500 ms; +57% ± 11% 60 min after tetanus, Fig. 3E).

Presynaptic Infusion of cADPR Antagonists Is Unable to Block LTD. Our previous studies indicate a probable presynaptic locus for NO-stimulated guanylyl cyclase (2), cGMP (2), and ryanodine-sensitive calcium stores (1), necessary for this form of LTD, suggesting that cADPR probably also acts presynaptically. To test this possibility directly, we infused either 8-Br-cADPR (1 mM) or 7-deaza-8-Br-cADPR (1 mM) into single CA1 pyramidal neurons by using intracellular microelectrodes. Fig. 4 illustrates the result of these experiments. After allowing 45–60 min for inhibitor to infuse into each neuron, we applied LFS (2 Hz/10 min; solid bars) to one of two Schaffer collateral inputs. Neither 8-Br-cADPR (Fig. 4A) nor 7-deaza-8-Br-cADPR (Fig. 4B) affected LTD when injected presynaptically, supporting the hypothesis that presynaptic cADPR receptors are the likely candidates for involvement in the induction cascade leading to LTD.

![Diagram](image-url)
DISCUSSION

This study supplies evidence for a physiologic role for the candidate messenger cADPR in mammalian long-term synaptic plasticity. We have shown (i) that hippocampal tissue possesses cyclase activity that can synthesize cADPR, (ii) that this molecule is, in fact, produced by elevating intracellular cGMP concentration in hippocampal slices, and (iii) that blockade of cADPR binding sites prevents the induction of LTD, but not LTP. However, we cannot entirely exclude the possibility that some other, closely related molecule that binds to cADPR-sensitive sites, on RyR or elsewhere, is actually the physiologic mediator of these actions.

The synthetic ADP-ribosyl cyclase and catabolic hydrolylase activities are usually colocalized on the same polypeptide, with hydrolylase activity exceeding cyclase. A notable exception is *Aplysia californica* ADP-ribosyl cyclase, whose extremely high activity and loose substrate specificity allowed synthesis of the candidate messenger in mammalian long-term synaptic plasticity. cADPR, including 8-Br-cADPR, a more potent, hydrolysis-resistant competitive inhibitor of cADPR binding sites. 7-deaza-cADPR yielded a much poorer substrate for cADPR hydrolylase. Combining the two substitutions produced 7-deaza-8-Br-cADPR, a more potent, hydrolysis-resistant competitive antagonist that selectively prevents cADPR-stimulated Ca2+ release (6). The potency and extent of blockade of LTD by each compound parallel nicely their efficacies, selectively preventing cADPR-sensitive Ca2+ release in many cells, including NO-stimulated Ca2+ release in neurons (9).

It should be noted that the phosphodiesterase V inhibitor zaprinast was effective in elevating cADPR concentration only when co-applied with the PKA inhibitor H89. This observation is intriguingly consistent with our recent finding (14) that these two inhibitors together induce an activity-dependent LTD of synaptic transmission at Schaffer collateral–CA1 synapses, whereas zaprinast alone causes only a reversible depression. Both these studies point to a bidirectional kinase regulation of synaptic plasticity, in which cGMP and cADPR are necessary but not sufficient for LTD, whereas cAMP and PKA promote LTP. Only when PKG is stimulated and PKA is inhibited is cADPR generated and LTD induced.

In Fig. 5, we propose a specific neuronal cascade for the selective induction of LTD. It is triggered by generation of the gaseous intercellular messenger NO in postsynaptic pyramidal neurons, and its diffusion to presynaptic Schaffer collateral terminals. NO stimulates guanylyl cyclase to produce cGMP and activate PKG. Among many possibilities, PKG causes stimulation of cADPR synthesis, which enhances RyR-mediated release of Ca2+ necessary to induce LTD. The fact that inhibition of NO-stimulated guanylyl cyclase, PKG, RyR, and cADPR activity all block induction of LTD supports this model.

Multiple observations support a presynaptic site for this cascade. Both postsynaptic cation of calcium (15) and extracellular cation of NO (7, 13) have been reported to prevent the induction of LTD, suggestive of diffusion of postsynaptically synthesized NO to a transsynaptic target. In inhibitor studies, postsynaptic injection of an NO-stimulated guanylyl cyclase inhibitor (2), PKG inhibitor (2), ryanodine (1), or cADPR antagonists all failed to block induction of LTD at Schaffer collateral–CA1 synapses, even though postsynaptic injection of dye (1) or other inhibitors (1, 2, 16) verified dendritic infusion. However, a study in dentate granule cells (17) has reported block of LTD by postsynaptic inhibition of the RyR antagonist ruthenium red, suggesting that different neuronal types may utilize differing cascades.

Other investigators have postulated a different cascade involving Ca2+-activated protein phosphatases (18), which they believe act postsynaptically (19). We propose that at least two distinctly, and computationally, different cascades exist on opposite sides of the synapse, separately responsible for altering presynaptic transmitter release and postsynaptic sensitivity, with both necessary for full expression of LTD (refs. 1 and 2; Fig. 5). Indeed, a recent report (20) has demonstrated that both N-methyl-D-aspartate and metabotropic glutamate receptor-dependent forms of LTD can be preferentially induced by different stimulus protocols, suggesting that different presynaptic activity patterns might trigger one form in preference to another.

It remains to be determined what targets are downstream of cADPR-triggered Ca2+ release. One candidate is presynaptic Ca2+/calmodulin-dependent protein kinase II (CaMKII). We have recently shown (16) that bath application of a membrane-permeant inhibitor of CaMKII prevents the induction of both LTD and LTP, whereas postsynaptic injection blocks only LTP. Clearly, synaptic vesicle proteins are appealing targets for both CaMKII and PKG. However, cADPR-mediated Ca2+ release has been shown to be calmodulin-dependent (21), and CaMKII has been shown to phosphorylate RyR directly (22), offering other possible targets of presynaptic regulation by CaMKII. Elucidation of the functional roles for cADPR in long-term synaptic plasticity is just beginning.

This work is dedicated to the memories of Lewis N. Stanton, Sr., and Vilma Maldonado. We thank A. Kyrozis, S. Nawy, T. Optiz, and A. Peinado for helpful discussions, and V. C. Bailey and G. A. Ashamu for ligand synthesis. This work was supported by Whitehall Foundation Grant A98-32 (to P.K.S.), Wellcome Trust Grant 045491 (to B.V.L.P.), and National Institutes of Health Medical Scientist Training Program Grant F31GM16379 (to M.R.H.).