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23 August 1999; accepted 29 October 1999

Enhanced Morphine Analgesia in Mice Lacking β-Arrestin 2

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The ability of morphine to alleviate pain is mediated through a heterotrimeric guanine nucleotide binding protein (G protein)–coupled heptahelical receptor (GPCR), the μ opioid receptor (μOR). The efficiency of GPCR signaling is tightly regulated and ultimately limited by the coordinated phosphorylation of the receptors by specific GPCR kinases and the subsequent interaction of the phosphorylated receptors with β-arrestin 1 and β-arrestin 2. Functional deletion of the β-arrestin 2 gene in mice resulted in remarkable potentiation and prolongation of the analgesic effect of morphine, suggesting that μOR desensitization was impaired. These results provide evidence in vivo for the physiological importance of β-arrestin 2 in regulating the function of a specific GPCR, the μOR. Moreover, they suggest that inhibition of β-arrestin 2 function might lead to enhanced analgesic effectiveness of morphine and provide potential new avenues for the study and treatment of pain, narcotic tolerance, and dependence.

GPCRs have important roles in mediating fundamental physiological processes such as vision, olfaction, cardiovascular function, and pain perception. Cellular communication through GPCRs requires the coordination of processes governing receptor activation, desensitization, and resensitization. However, the relative contribution of desensitization mechanisms to the overall homeostatic process still remains largely unexplored in vivo. GPCR kinases (GRKs) act to phosphorylate activated receptors and promote their interaction with β-arrestins. This, in turn, prevents further coupling with G proteins and disrupts normal activation of the second messenger signaling cascade. By this mechanism, GRKs and β-arrestins can act to dampen GPCR signaling, thereby leading to desensitization of the receptor (*1*). At least six GRKs (GRK1 to GRK6) and four arrestins (visual and cone arrestin, β-arrestins 1 and 2) have been discovered; however, the functional importance of such redundancy is unclear. Overexpression (*2*) or inactivation (*3*) of certain GRKs leads to modulation of receptor responsiveness. In addition, mice that are deficient in β-arrestin 1 display increased cardiac contractility in response to β adrenergic receptor agonists (*4*). Therefore, the use of animal models in which the genes for GRKs and β-arrestins are functionally inactivated should help to elucidate the contribution of the desensitization mechanisms to the physiological responses.

Because GPCRs, such as the substance P receptor and the opioid receptors, participate in processing the sensation of pain, we characterized analgesic responses through the μ opioid receptor (μOR) in mice lacking β-arrestin 2. In the clinical setting, morphine is currently the most effective drug for alleviating intense and chronic pain. The antinociceptive (blocking of pain perception) actions of morphine are mediated through stimulation of the μOR, as demonstrated by the lack of morphine analgesia observed in knockout mice deficient in the μOR (*5, 6*). Nevertheless, the neuronal signaling mechanisms mediating analgesia through μORs and morphine remain poorly understood. Moreover, the contribution of GPCR desensitization to the onset and duration of analgesia has been unclear.

We generated β-arrestin 2 knockout (βarr2-KO) mice by inactivation of the gene by homologous recombination (*7*). Mice lacking β-arrestin 2 were identified by Southern (DNA) blot analysis (Fig. 1A), and the absence of β-arrestin 2 was confirmed by protein immunoblotting of extracts from brainstem, periaqueductal gray (PAG) tissue, spleen, lung, and skin (Fig. 1B) (*8*). Because wild-type, heterozygous ($\beta arr2^{+/-}$), and homozygous mutant mice had similar amounts of β-arrestin 1 in the brain regions examined (Fig. 1B), compensatory upregulation of β-arrestin 1 in the absence of β-arrestin 2 seems unlikely. The βarr2-KO mice were viable and had no gross phenotypic abnormalities. However, after administration of morphine, obvious differences became apparent between the genotypes.

Morphine-induced antinociception was evaluated by measuring response latencies in the hot-plate test. We used a dose of morphine (10 mg/kg body weight) and route of administration (subcutaneous) that are known to induce analgesia in many strains of mice (*9*). The analgesic effect of morphine was significantly potentiated and prolonged in the knockout mice relative to their wild-type littermates (Fig. 2). Such robust responses to morphine were absent not only in the wild-type littermates (Fig. 2) but also in the parental mouse strains (C57BL/6 and 129SvJ) used to generate this knockout (*10*). Four hours after the morphine injection, βarr2-KO mice still exhibited significant analgesia [percent maximum possible effect (MPE) = 31 ± 0.4%], whereas in their wild-type littermates, the analgesic effects of the same dose of morphine waned after about 90 min. $\beta arr2^{+/-}$ mice were nearly as responsive to morphine as the βarr2-KO mice; however, this may reflect the imposed limit of the hot-plate assay (30 s), which is designed to prevent prolonged exposure of the mice to pain. Basal responses to the hot plate did not differ between genotypes (wild type, 6.2 ± 0.3 s, $n = 25$; βarr2-KO, 6.1 ± 0.4 s, $n = 27$). The differences in morphine-induced analgesia between the genotypes are unlikely to

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be due to pharmacokinetic differences in morphine metabolism, because the concentrations of morphine in blood, as determined by mass spectroscopy analysis, did not differ between wild-type and βarr2-KO mice 2 hours after morphine injection (*11*).

Lower doses of morphine were also tested in these assays. Even at doses of morphine that were subanalgesic in wild-type mice [1 mg/kg subcutaneously (sc)], βarr2-KO animals displayed a significant increase in their nociceptive thresholds (Fig. 3). At 30-min intervals, immediately after the antinociception test, mice were given repeated cumulative doses of morphine resulting in final concentrations of 5 and 10 mg/kg (*6*). At the highest cumulative dose, mice reached levels of antinociception similar to that seen in Fig. 2, in which the same amount of morphine was administered in a single injection. At every dose, the βarr2-KO animals experienced greater antinociception after morphine treatment than did their wild-type littermates.

To test whether the analgesic effects of morphine were mediated by actions at the μOR, we treated mice with various antagonists (*12*). Naloxone, a well-established OR antagonist, was administered to the same mice immediately after measuring the antinociceptive effects of morphine (10 mg/kg). Naloxone (2.5 mg/kg sc) completely reversed the effects of morphine in both the wild-type and βarr2-KO animals within 10 min. However, the δ and κ OR-selective antagonists naltrindole (2.5 mg/kg sc) and norbinaltorphimine (5 mg/kg sc) did not inhibit analgesia in either the wild-type or βarr2-KO mice (*10*). The morphine dose dependency of the antinociceptive response and the reversal of the effects with naloxone suggest that the potentiated and prolonged effects in mice that lack β-arrestin 2 result from stimulation of the μOR.

Wild-type and βarr2-KO mice were also evaluated for changes in body temperature (*13*). No significant differences in basal body temperature were found between genotypes; however, the βarr2-KO mice experienced a greater drop in body temperature after morphine treatment than did the wild-type mice (Fig. 4). This decrease in temperature also persisted longer than that in their wild-type littermates.

To investigate whether the μOR population was altered in the KO mice, we performed radioligand binding analysis on membranes (*14*) prepared from different brain regions (Table 1). Saturation binding studies with [^{3}H]naloxone, at concentrations that preferentially label the μOR, revealed a single high-affinity binding site, which represents the μOR (*15*). Hypothalamus, brainstem, and PAG regions were chosen because they contain μORs and are implicated in the regulation of pain and body temperature (*16*). The number and affinity of μORs did not significantly differ between the two genotypes in any of the brain regions examined.

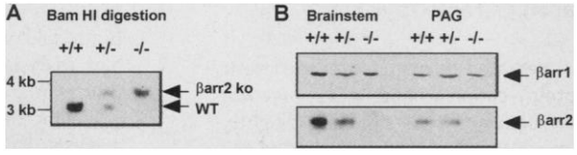

Fig. 1. Characteristics of the targeted disruption of the mouse β-arrestin 2 (βarr2) gene. **(A)** Southern blot analysis of genomic DNA from wild-type (WT), heterozygous (+/−), and homozygous (−/−) mice. Tail DNA was digested with Bam HI and analyzed by Southern blotting with the 5′ probe. A 3.5-kb fragment is indicative of the βarr2-KO allele, and a 3-kb fragment is indicative of the wild-type allele. **(B)** Protein immunoblot analysis of βarr2 expression in WT, βarr2$^{+/-}$, and βarr2-KO mice. Membranes were blotted for βarr1 (top) and βarr2 (bottom) protein expression. Each lane was loaded with 25 μg of protein derived from the same lysates of the indicated brain regions.

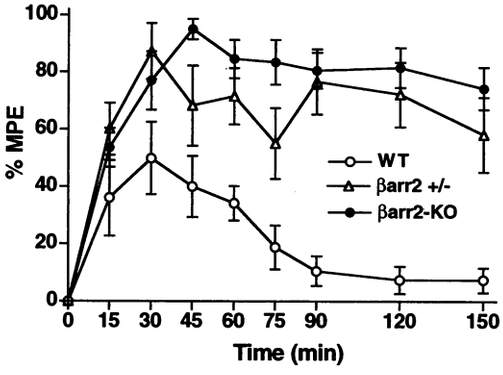

Fig. 2. Enhanced and prolonged morphine-induced antinociception in βarr2-KO mice. Antinociceptive responses were measured as hot-plate response latency (56°C) after morphine treatment (10 mg/kg sc) (*8*). The "response" was defined by the animal either licking the fore- or hindpaws or flicking the hindpaws. In these studies, the most prominent response was forepaw licking. To avoid tissue damage, we exposed the animals to the plate for a maximum of 30 s. Data are reported as percentages of this maximum response time, as determined from each individual mouse's basal response, the response after drug treatment, and the imposed maximum cutoff time with the following calculation (*9*): 100% × [(drug response time−basal response time)/(30 s−basal response time)] = % maximum possible effect (% MPE). WT (n = 6), heterozygous (+/–, n = 5), and KO (n = 9) mice were analyzed together in the same experiment. The % MPE curves of the βarr2-KO and βarr2$^{+/-}$ mice were significantly greater than the WT response curve ($P < 0.001$), as determined by two-way analysis of variance (ANOVA).

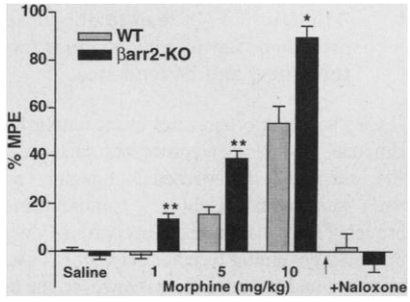

Fig. 3. Greater dose-dependent antinociceptive responses to morphine in βarr2-KO mice. The degree of antinociception was determined by measuring the latency of hot-plate responses (Fig. 2). Withdrawal latencies were measured 30 min after a first dose of morphine (1 mg/kg sc); at this time point, animals were injected with morphine (4 mg/kg sc) for a cumulative dose of 5 mg/kg. Antinociception was again assessed after 30 min, and mice were again injected with morphine (5 mg/kg sc) to yield a final cumulative dose of 10 mg/kg. Withdrawal latencies were again measured after 30 min, and the mice were then injected with naloxone (2.5 mg/kg sc). After 10 min, antinociception was assessed once more. WT (n = 7) and βarr2-KO (n = 6) mice were significantly different at each dose (*$P < 0.01$, **$P < 0.001$; Student's t test). Means ± SEM are shown. In an additional experiment, morphine (25 mg/kg sc) induced the maximum imposed response (100%) in both genotypes (*10*). Thus, an approximate twofold difference in apparent ED_{50} was observed between genotypes [WT, 9.77 (8.08 to 11.81) mg/kg; KO, 5.98 (5.10 to 6.94) mg/kg (95% confidence intervals)].

Table 1. [^{3}H]Naloxone binding in brain regions of WT and KO mice. Saturation binding assays were performed on membranes (*12*) from different brain regions (50 to 100 μg per tube) with increasing concentrations of [^{3}H]naloxone (0 to 12 nM, 52.5 Ci/mmol; Amersham). Nonspecific binding was determined in the presence of 10 μM naloxone. Membranes were incubated at 25°C for 1 hour. Binding parameters were determined by Scatchard analysis of specific binding. Data are means ± SEM of three or four experiments performed in duplicate. B_{max}, maximum binding capacity; K_D, dissociation constant.

Brain region	WT		βarr2-KO	
	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)
PAG	132 ± 9	4.0 ± 0.1	144 ± 13	4.5 ± 0.8
Brainstem	49 ± 7	1.5 ± 0.2	54 ± 9	3.0 ± 0.8
Hypothalamus	103 ± 18	6.2 ± 1.6	89 ± 8	3.8 ± 0.2

Additional evidence for increased sensitivity of the μOR in βarr2-KO animals was obtained in biochemical experiments. We measured agonist-stimulated binding of [^{35}S]guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S) to G proteins in isolated membranes, the most proximal manifestation of GPCR activation (*17*). Because morphine acts in vitro to stimulate μ, δ, and κ opioid receptors, the μOR-selective agonist [D-Ala^2, MePhe^4, Gly^5-*ol*]enkephalin (DAMGO) was used to specifically activate G protein coupling to μORs. DAMGO stimulated more [^{35}S]GTP-γ-S binding in membranes derived from βarr2-KO mice than in those derived from wild-type littermates (Fig. 5). Similar results were also obtained in brainstem membranes (*10*). Amounts of Gα protein ($G_{i/o/z}$), as determined by protein immunoblotting, did not vary between the genotypes (*10*). These observations suggest that enhanced coupling of μORs to G proteins took place in tissues derived from βarr2-KO mice. Although the enhanced analgesia induced by morphine may involve complex neurological signaling, this biochemical evidence supports the interpretation that the enhanced physiological responsiveness in the knockout animals results from increased sensitivity of signaling by the μOR.

In transfected cultured cells, the degree of β_2 adrenergic receptor signaling is dependent on the cellular complement of GRK2 and GRK3 (*18*) and β-arrestins (*18, 19*). These observations, along with those presented here, directly support the proposed role of β-arrestin 2 in preventing further receptor–G protein coupling and mediating desensitization of the GPCR. Moreover, β-arrestins not only are involved in the dampening of GPCR responsiveness after agonist stimulation, but also influence the sensitivity of the response.

The simplest interpretation of these results is that μOR signaling is regulated by β-arrestin 2. However, in transfected cells, morphine fails to induce the internalization of the μOR (*20, 21*), and a green fluorescent protein–tagged β-arrestin 2 fails to translocate to μOR overexpressed in cell culture upon exposure to morphine (*21*). These in vitro studies were conducted with the rat μOR or the mouse μOR (MOR1), which are not particularly rich in potential phosphorylation sites. Several splice variants of the μOR are present in mouse brain that contain several potential phosphorylation sites (*22*). Some of these isoforms can contribute to morphine-induced analgesia. The involvement of these receptors might explain the differences between the in vitro studies and those with the βarr2-KO mice.

The βarr2-KO mice were similar in phenotype to their wild-type littermates, and other GPCR-directed drugs did not necessarily elicit different responses between the genotypes. For example, locomotor responses to dopamine receptor stimulation by cocaine and apomorphine were not enhanced (*23*). These observations suggest that various GPCRs are differentially affected by the loss of β-arrestin 2. Other regulatory elements, such as GRKs or β-arrestin 1, could compensate for the lack of β-arrestin 2, or the receptors could vary in their requirement for β-arrestin interaction for their regulation.

Our studies demonstrate that the absence of β-arrestin 2 can affect the efficacy of GPCR activation in an animal model, and that the enhancement of opiate analgesia through selective alterations of GPCR signaling components may have therapeutic potential.

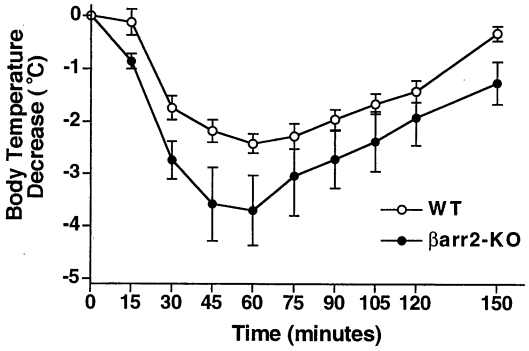

Fig. 4. Increased hypothermic responses to morphine in βarr2-KO mice. Rectal body temperatures were measured with a digital thermometer (*13*). Basal body temperatures did not vary significantly between genotypes (WT, 36.4 ± 0.1°C; KO, 36.8 ± 0.1°C). WT (n = 5) and KO (n = 5) animals were analyzed in parallel during the same experiment. The curves are significantly different (P < 0.001) as determined by two-way ANOVA. Means ± SEM are shown.

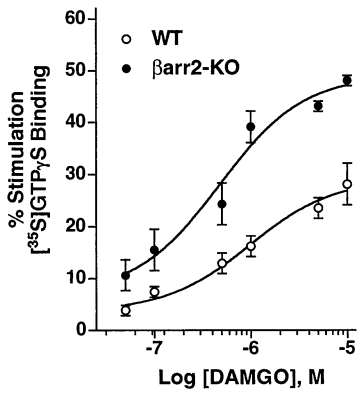

Fig. 5. Binding of [^{35}S]GTP-γ-S to PAG membranes from WT and βarr2-KO mice. [^{35}S]GTP-γ-S binding to isolated PAG membranes (*12*) was determined after 2 hours of stimulation (at 30°C) with 50 to 10,000 nM DAMGO. PAG membranes (10 μg of protein per assay tube) were incubated in the presence of 10 μM GDP and 50 pM [^{35}S]GTP-γ-S (1250 Ci/mmol; NEN, Boston). [^{35}S]GTP-γ-S binding was measured as described (*15*). [^{35}S]GTP-γ-S binding is expressed as percent increase in [^{35}S]GTP-γ-S binding relative to binding in unstimulated samples. Data were analyzed by nonlinear regression using GraphPad Prism software and are presented as means ± SEM of at least three experiments performed in triplicate wherein WT and βarr2-KO brain regions were assayed simultaneously. In the absence of agonist stimulation, basal [^{35}S]GTP-γ-S binding was 440 ± 83 cpm for WT mice and 527 ± 99 cpm for βarr2-KO mice.

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7. A bacteriophage λ library of mouse 129SvJ genomic DNA (Stratagene) was screened with the rat βarr2 cDNA (*24*). Positive phages were identified and analyzed by restriction endonuclease digestion. A 12-kb βarr2 fragment was digested with Bam HI, subcloned into pBluescript KS(-), and sequenced. The targeting vector was assembled by blunt-end ligation of a pHSV-TK cassette (from plasmid pIC19R/MCI-TK), a 2.8-kb Nco I–Bam HI βarr2 fragment, a pGK-neo cassette (from plasmid pD383) that replaced the 0.8-kb Bam HI–Hind III fragment of βarr2, and a 4.5-kb Hind III βarr2 fragment into pBluescript KS(-). This targeting vector was linearized with Not I and was electroporated into mouse embryonic stem cells. Genomic DNA from transfectants resistant to G418 and gancyclovir were isolated and screened by Southern blot analysis using a 0.2-kb 5′ external βarr2 probe and a 0.3-kb 3′ external βarr2 probe. Supplemental information regarding the targeting construct is available at *Science* Online (www.sciencemag.org/feature/data/1045481.shl). Chimeric animals were generated by microinjecting these embryonic stem cells into C57BL/6 blastocysts. Five chimeric male pups were obtained and mated with C57BL/6 females. Germ line transmission was confirmed by Southern blotting. Heterozygous offspring were intercrossed to obtain homozygous mice. Wild-type and mutant mice used in this study were age-matched, 3- to 5-month-old male siblings. For protein immunoblot analysis, whole-cell lysates were prepared by polytron homogenization in lysing buffer [10 mM tris (pH 7.4), 5 mM EDTA, one protease inhibitor tablet per 10 ml (Roche Molecular Biochemicals, Indianapolis, IN), and 1% Nonidet-40]. Polyacrylamide gels were loaded with 25 μg of protein per lane, and equivalent protein loading was confirmed by Ponceau S staining of the gels. After transfer to polyvinyldifluoride membranes, proteins were blotted with polyclonal antibodies to β-arrestin 2 or β-arrestin 1 (*24*). Bands were visualized with secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence detection system (Amersham). All experiments were conducted in accordance with the NIH guidelines for the care and use of animals and with an approved animal protocol from the Duke University Animal Care and Use Committee.
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11. Mice were injected with morphine (10 mg/kg sc). After 30 min or 2 hours, wild-type mice were killed and blood was collected in vials containing sodium fluoride and potassium oxalate. Morphine concentrations in blood samples pooled from three mice per sample were 1500 ng/ml after 30 min and 83 ng/ml after 2 hours, as measured by mass spectroscopy analysis (Occupational Testing Division, LabCorp Inc., Research Triangle Park, NC). In similar experiments, βarr2-KO mice had a concentration of 93 ng/ml in the blood after 2 hours.
12. Naloxone (2.5 mg/kg sc), which immediately reverses the effects of opiates, was given 30 min after morphine (10 mg/kg). Naltrindole [P. S. Portoghese, M. Sultana, A. E. Takemori. *J. Med. Chem.* **88**, 1547 (1990)] was given 20 min before morphine, and nor-binaltorphimine [A. E. Takemori, B. Y. Ho, J. S. Naeseth, P. S. Portoghese, *J. Pharmacol. Exp. Ther.* **246**, 255 (1988)] was given 1 hour before morphine [H. W. Matthes *et al.*, *J. Neurosci.* **18**, 7285 (1998)].
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14. Membranes were prepared for binding assays as follows. Brain regions were dissected and immediately frozen in liquid nitrogen and were stored at −80°C for less than 1 week before use. Samples were placed on ice and homogenized by polytron in membrane preparation buffer [50 mM tris (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$], and crude membranes were prepared by centrifugation at 20,000*g* for 15 min at 4°C. Membranes were resuspended in either 50 mM tris-HCl (pH 7.4) for radioligand binding assays or in assay buffer [50 mM tris-HCl (pH 7.4), 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM EDTA] containing 10 μM guanosine diphosphate for [^{35}S]GTP-γ-S binding assays. For both binding assays, reactions were terminated by rapid filtration over GF/B filters (Brandel Inc., Gaithersburg, MD) using a Brandel cell harvester. Filters were washed three times with ice-cold 10 mM tris-HCl (pH 7.4) and then counted in a liquid scintillation counter.
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25. We thank B. H. Koller for homologous recombinant mouse embryonic stem cells and chimeric mice, M. R. Capecchi for plasmid pIC19R/MCI-TK, and R. Hen for plasmid pD383. R.R.G. is a visiting scientist from the Institute of Pharmacology, Russian Academy of Medical Sciences, Moscow. Supported in part by NIH grants NS 19576 (M.G.C.) and HL16037, and by a cardiovascular unrestricted grant from Bristol-Myers Squibb (R.J.L.). R.J.L. and M.G.C. are Investigators of the Howard Hughes Medical Institute.

16 September 1999; accepted 22 November 1999

Induction of Nitric Oxide–Dependent Apoptosis in Motor Neurons by Zinc-Deficient Superoxide Dismutase

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Mutations in copper, zinc superoxide dismutase (SOD) have been implicated in the selective death of motor neurons in 2 percent of amyotrophic lateral sclerosis (ALS) patients. The loss of zinc from either wild-type or ALS-mutant SODs was sufficient to induce apoptosis in cultured motor neurons. Toxicity required that copper be bound to SOD and depended on endogenous production of nitric oxide. When replete with zinc, neither ALS-mutant nor wild-type copper, zinc SODs were toxic, and both protected motor neurons from trophic factor withdrawal. Thus, zinc-deficient SOD may participate in both sporadic and familial ALS by an oxidative mechanism involving nitric oxide.

Only 2% of ALS patients carry 1 of 60 different dominant mutations in Cu,Zn SOD, raising questions about how SOD might be involved in the majority of sporadic ALS patients expressing only wild-type (WT) SOD (*1*). Transgenic mouse experiments establish that ALS mutations increase a toxic gain-of-function of the protein that kills motor neurons (*2*). SOD is the major defense against superoxide ($O_2^{\cdot-}$), but WT SOD does not protect against the toxicity of ALS-SOD in mice or humans (*1*, *3*). Curiously, ALS-SODs can scavenge superoxide as efficiently as WT SOD when they contain their full complement of Cu and Zn (*4*). However, the mutations destabilize the SOD protein, which indirectly decreases Zn affinity by 5- to 50-fold compared with WT SOD (*5*, *6*). Mutations such as A4V (substitution of alanine at position 4 by valine), which cause rapid disease progression, yield SODs with the weakest affinity for Zn. Because Zn deficiency diminishes superoxide scavenging and increases tyrosine nitration by SOD (*6*), we examined how the altered redox properties of Zn-deficient SOD could be toxic to cultured motor neurons.

Loss of Zn visibly changed SOD from a "green" to a "blue" protein by altering the coordination of Cu through a shared histidine ligand (Fig. 1A). Reduction of the Cu to Cu^+ makes both forms of SOD colorless. The altered Cu coordination made Zn-deficient SOD a more efficient oxidant, able to oxidize ascorbate 3000-fold faster than Cu,Zn SOD, irrespective of whether the protein was WT or carried an ALS mutation (Fig. 1B). Other cellular reductants such as urate, glutathione, and cysteine could also reduce Zn-deficient SOD, but more slowly than ascorbate. Because such reduced SOD is slowly reoxidized by oxygen (*7*), Zn-deficient SOD can generate superoxide at the expense of cellular antioxidants. Cells still contain high concentrations of Cu,Zn SOD (*8*), which will quickly recapture superoxide leaking from reduced Zn-deficient SOD. However, nitric oxide (NO) can effectively compete with SOD for superoxide to produce the strong oxidant peroxynitrite ($ONOO^-$) (*9*). Peroxynitrite formation was assayed with the indicator dichlorodihydrofluorescein (DCDHF), which is oxidized by peroxynitrite but not by NO, superoxide, or hydrogen peroxide (H_2O_2) (*10*). Ascorbate plus Zn-deficient SOD oxidized DCDHF only in the presence of NO and under aerobic conditions (Fig. 1C). Adding a 1.5-fold excess of Zn to Zn-deficient SOD prevented DCDHF oxidation, showing that the Zn-deficient SOD can rapidly bind Zn to behave like Cu,Zn SOD in vitro. Oxidation of DCDHF did not involve H_2O_2 because catalase had no effect and addition of 20 μM H_2O_2 to Zn-deficient SOD did not oxidize DCDHF.

Importantly, WT Cu,Zn SOD did not pre-

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