Role of β-Arrestin in Mediating Agonist-Promoted G Protein–Coupled Receptor Internalization

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β-Arrestins are proteins that bind phosphorylated heterotrimeric GTP-binding protein (G protein)–coupled receptors (GPCRs) and contribute to the desensitization of GPCRs by uncoupling the signal transduction process. Resensitization of GPCR responsiveness involves agonist-mediated receptor sequestration. Overexpression of β-arrestins in human embryonic kidney cells rescued the sequestration of β_2-adrenergic receptor (β_2AR) mutants defective in their ability to sequester, an effect enhanced by simultaneous overexpression of β-adrenergic receptor kinase 1. Wild-type β_2AR sequestration was inhibited by the overexpression of two β-arrestin mutants. These findings suggest that β-arrestins play an integral role in GPCR internalization and thus serve a dual role in the regulation of GPCR function.

β-arrestin proteins contribute to the regulation of GPCR responsiveness by binding G protein–coupled receptor kinase–phosphorylated (for example, β-adrenergic receptor kinase 1, βARK1) receptors and uncoupling the receptors from their heterotrimeric G proteins (*1–4*). This process, called desensitization (*5*), is agonist-dependent and renders GPCRs less responsive to additional stimulation. Therefore, continued cell signaling through GPCRs requires their reactivation. Agonist-promoted sequestration (internalization) of GPCRs is thought to contribute to the functional resensitization of their responsiveness, likely as a result of their dephosphorylation in endosomes (*6*). The molecular intermediates directing this process are not well characterized, but βARK1-mediated phosphorylation facilitates both β_2AR and m2 muscarinic acetylcholine receptor sequestration (*7, 8*). Because βARK phosphorylation promotes the interaction of arrestin proteins with GPCRs in vitro (*2, 9*), we tested whether β-arrestins might participate in the GPCR sequestration process. Our results establish that β-arrestin proteins do indeed play a role in the sequestration of the β_2AR and suggest that they might act as adaptor-like molecules in this process. Because GPCRs mediate the activity of a variety of signaling processes, such as neurotransmission, hormonal response, olfaction, and light transduction (*10–13*), these findings suggest that β-arrestins may play a more dynamic role in the regulation of biological functions.

Initial experiments examined the role of β-arrestin proteins in GPCR sequestration by testing their ability to rescue the sequestration phenotype of β_2AR mutants defective in their sequestration. Under control conditions, agonist stimulation resulted in the internalization of 30 ± 3.1% of transfected wild-type β_2ARs (Fig. 1A). Truncation of the COOH-terminal tail of the β_2AR at cysteine residue 341 to remove putative βARK phosphorylation sites (T^{341}) (*14*) or mutation of putative sites for βARK- and PKA-mediated β_2AR phosphorylation ($Phos^-$) (*3*) impaired agonist-promoted β_2AR sequestration by 50% (Fig. 1A). Overexpression of either β-arrestin–1 or –2 subtype rescued the sequestration phenotype of the truncated and phosphorylation site–deficient β_2AR mutants, whereas βARK1 overexpression did not rescue their sequestration (Fig. 1A).

Mutation of tyrosine residue 326 to an alanine results in a β_2AR mutant (β_2AR-Y326A) that is both deficient in its agonist-promoted sequestration and βARK-mediated phosphorylation, and overexpression of βARK1 reverses both the sequestration and phosphorylation deficits (*7*). Similar to βARK1, overexpression of either β-arrestin subtype rescued the sequestration of the β_2AR-Y326A mutant (Fig. 1B). However, unlike βARK1, overexpression of either β-arrestin–1 or –2 rescued the sequestration deficits of both the truncated and phosphorylation site–deficient β_2AR-Y326A mutants (Fig. 1B). Thus, when overexpressed to high levels, β-arrestins interacted with the receptor mutants in the absence of phosphorylation. Data from in vitro experiments suggest that β-arrestins can bind with lower affinity to agonist-activated nonphosphorylated receptor (*2, 9*).

If the ability of βARK1 overexpression to rescue the sequestration of the β_2AR-Y326A mutant was to provide a phosphorylated receptor with which endogenous β-arrestins could interact, then one might expect that β-arrestins could rescue the sequestration of the mutant receptor more effectively in the presence of low levels of βARK1 overexpression. Thus, we examined the effect of 0, 0.1, and 0.25 μg of βARK1 complementary DNA (cDNA) cotransfected with increasing amounts of β-arrestin–2 cDNA on the rescue of β_2AR-Y326A mutant sequestration. Cotransfection of HEK 293 cells with 0.1 and 0.25 μg of βARK1 cDNA shifted the dose-response curve for β-arrestin–2–mediated rescue of β_2AR-Y326A mutant sequestration by 3.9- and 10-fold to the left, respectively. In the absence of cotransfected β-arrestin, increased βARK1 levels

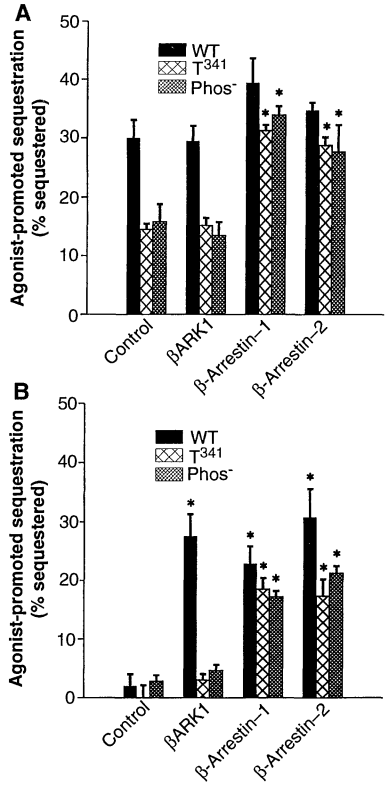

Fig. 1. Effect of βARK1, β-arrestin–1, and β-arrestin–2 overexpression (*18*) on the agonist-promoted sequestration (*19*) of wild-type (WT), Cys^{341} truncated (T^{341}), or PKA- and βARK phosphorylation site–deficient ($Phos^-$) β_2AR (**A**) and similarly mutated β_2AR-Y326A (**B**) transfected transiently (*20*) into HEK 293 cells. Receptors were expressed with a pcDNA1-Amp expression vector together with 5 μg of each of the following: empty pcMV5 vector (control), pcDNA1 bovine βARK1, pcMV5 rat β-arrestin–1, or pcMV5 rat β-arrestin–2. Receptor expression (fmol/mg whole-cell protein) was as follows: WT-β_2AR, 687 ± 77; β_2AR-T^{341}, 264 ± 22; β_2AR-$Phos^-$, 161 ± 25; WT-β_2AR-Y326A, 307 ± 45; β_2AR-Y326A-T^{341}, 97 ± 8; and β_2AR-Y326A-$Phos^-$, 440 ± 47 (*21*). The data represent the mean ± SEM of four to six different experiments. *$P < 0.05$ compared with control values.

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resulted in a progressive rescue of agonist-promoted β_2AR-Y326A mutant sequestration, presumably as a result of enhanced interaction of phosphorylated mutant receptor with endogenous β-arrestins. The shift in the half-maximal effective concentration (EC_{50}) (Fig. 2) for β-arrestin–mediated rescue of β_2AR-Y326A mutant sequestration in the presence of βARK1 overexpression indicated that the role played by βARK phosphorylation in facilitating β_2AR sequestration was to promote the interaction of the receptor with β-arrestin proteins.

In addition to β-arrestin–1 and β-arrestin–2, two β-arrestin mutants (β-arrestin–1–V53D and β-arrestin–2–V54D) (*15*) were expressed and tested for their ability to influence sequestration (Fig. 3). The valine residue substituted for an aspartic acid in both β-arrestin–1 and β-arrestin–2 is found at the beginning of an amino acid sequence conserved among all the members of the arrestin family and is analogous to a mutation described in mutagenesis studies of *Drosophila* arrestins, arrestin-2–V52D (*11*). Expression of this mutant arrestin could not be detected in the *Drosophila* system (*11*), but both the β-arrestin–1–V53D and the β-arrestin–2–V54D proteins could be overexpressed in HEK 293 cells (Fig. 3A). β-arrestin–1–V53D inhibited wild-type β_2AR sequestration in an expression-dependent manner by as much as 75 ± 9% but did not rescue the sequestration of the β_2AR-Y326A mutant (Fig. 3B). A similar result was obtained for β-arrestin–2–V54D (Fig. 3C). The kinetics of β_2AR sequestration were examined in the absence or presence of 5 μg of cotransfected β-arrestin–1 or β-arrestin–1–V53D to test whether the effect of the mutant β-arrestin was to decrease either the rate or the extent of β_2AR internalization (Fig. 3D). Whereas β-arrestin–1 had no effect, β-arrestin–1–V53D reduced the rate of β_2AR internalization, as measured by the slope of the curve at $t = 0$ by 50 to 70%, suggesting that the mutant protein competes with endogenous β-arrestins, thus acting as a "dominant-negative" with respect to sequestration (*16*).

Because the rescue of sequestration by βARK has been associated with rescued phosphorylation of the β_2AR-Y326A mutant (*7*), we sought to eliminate the possibility that the inhibitory effect of β-arrestin–1–V53D was caused by its interference with receptor phosphorylation. Under control conditions, the β_2AR-Y326A mutant did not serve as an effective substrate for phosphorylation, because it was phosphorylated to 30 ± 7% of the level achieved with the wild-type β_2AR (Fig. 4A) (*7*). β_2AR-Y326A mutant phosphorylation was rescued by overexpression of βARK1, even in the presence of β-arrestin–1–V53D, but was unaffected by either β-arrestin–1 or β-arrestin–1–V53D alone (Fig. 4A). Phosphorylation of the wild-type β_2AR remained unchanged after transfection with β-arrestin–1, whereas transfection with βARK1, β-arrestin–1–V53D, or βARK1 with β-arrestin–1–V53D resulted in increased levels of wild-type β_2AR phosphorylation (Fig. 4A). The increased level of wild-type β_2AR phosphorylation observed after cotransfection with β-arrestin–1–V53D was likely due to reduced receptor sequestration that, if involved in receptor dephosphorylation (*17*), would be expected to result in an increase in the apparent number of phosphorylated receptors.

Because overexpression of βARK1 rescued β_2AR-Y326A mutant phosphorylation in the presence of overexpressed β-arrestin–1–V53D, the ability of βARK1 to rescue β_2AR-Y326A mutant sequestration under similar conditions was tested. βARK1 coexpressed with β-arrestin–1–V53D could not rescue the sequestration of the β_2AR-Y326A mutant, whereas it was able to rescue β_2AR-Y326A sequestration when overexpressed in the absence of mutant β-arrestin (Fig. 4B). Wild-type β_2AR sequestration was inhibited by β-arrestin–1–V53D even in the presence of overexpressed βARK1 (Fig. 4B). Thus, βARK phosphorylation alone would not be sufficient to rescue the sequestration of the β_2AR-Y326A mutant in the absence of endogenously expressed β-arrestin proteins.

The ability of the β-arrestin–1–V53D and β-arrestin–2–V54D mutants to inhibit

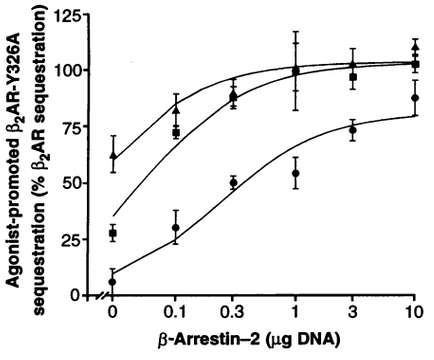

Fig. 2. Effect of βARK1 on the rescue of β_2AR-Y326A sequestration by β-arrestin–2. The β_2AR-Y326A mutant was cotransfected with increasing amounts of pcMV5 β-arrestin–2 cDNA (0 to 10 μg) in the absence (●) or presence of either 0.1 (■) or 0.25 (▲) μg pcDNA1 βARK1 cDNA. The data represent the mean ± SEM of three different experiments. β_2AR and β_2AR-Y326A expression levels were 1269 ± 500 and 977 ± 370 fmol/mg whole-cell protein, respectively, and wild-type β_2AR sequestration was 26 ± 1%. βARK1 was overexpressed 2.5- and 7-fold above endogenous expression levels with 0.1 and 0.25 μg of βARK1 cDNA, respectively. β-Arrestin–2 was overexpressed from 4- to 100-fold above endogenous levels of β-arrestin–1 and –2 expression as assessed by densitometric analysis of immunoblots (*7, 12, 13*). The curves were fit and analyzed with GraphPad prism. The EC_{50} values for β-arrestin–2 expression–dependent promotion of β_2AR-Y326A sequestration were 0.22 ± 0.07, 0.06 ± 0.02, and 0.02 ± 0.01 μg of pcMV5 β-arrestin–2 cDNA in the absence or presence of either 0.1 or 0.25 μg of cotransfected pcDNA1 βARK1 cDNA, respectively.

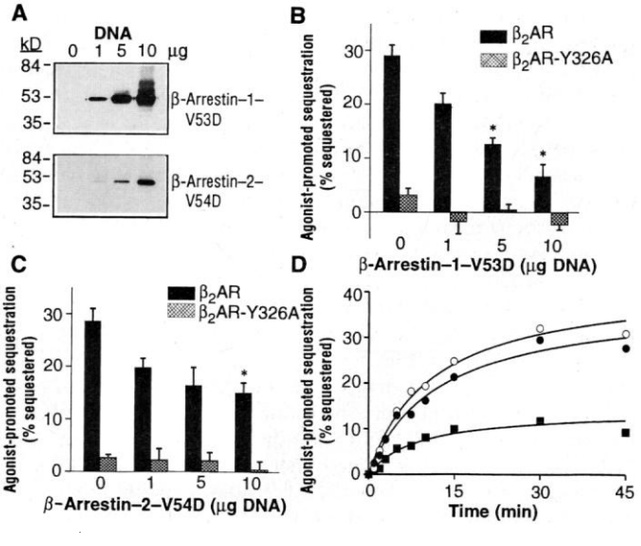

Fig. 3. Inhibition of wild-type β_2AR sequestration by β-arrestin–1 and β-arrestin–2 mutants. (**A**) Immunoblot demonstrating the overexpression of β-arrestin–1–V53D and β-arrestin–2–V54D mutant proteins with increasing amount of transfected cDNA as detected with an antibody cross-reactive for both β-arrestin–1 and –2 (*7, 13*). In (B) and (C), HEK 293 cells were transfected transiently with pcDNA1-Amp encoding the cDNA for either the wild-type β_2AR or β_2AR-Y326A mutant together with 0, 1, 5, or 10 μg of pcDNA1-Amp encoding the cDNA for either (**B**) β-arrestin–1–V53D or (**C**) β-arrestin–2–V54D and tested for agonist-promoted sequestration. The data represent the mean ± SEM of four different experiments. *$P < 0.05$ compared with control values. (**D**) The time course for β_2AR sequestration in the absence (●) or presence of either 5 μg of pcMV5 β-arrestin–1 (○) or 5 μg of pcDNA1-Amp β-arrestin–1–V53D (■). Each data point represents the mean from four different experiments. The kinetic curves were fit with GraphPad Prism. In (B) and (C), β_2AR and β_2AR-Y326A expression levels were 1120 ± 80 and 1310 ± 146 fmol/mg whole-cell protein, and in (D), β_2AR expression was 1244 ± 229 fmol/mg whole-cell protein.

wild-type β_2AR sequestration in a competitive fashion argues for their function as "dominant-negative" β-arrestins with respect to sequestration. Several mechanisms might explain this phenotype. The mutant β-arrestins might act as protein "sinks," such that they are unable to bind to the receptor but are instead constitutively bound to a cellular component required for receptor internalization. Alternatively, because the actions of β-arrestin–1–V53D appear to be competitive with respect to β_2AR sequestration, the mutant β-arrestins might interact normally with the receptors but fail to mediate subsequent interactions with other proteins required for receptor sequestration and thus diminish the pool of receptors exhibiting the capacity to sequester. In either case, the data suggest that β-arrestins act as adaptor-like molecules, which serve either to recruit cellular proteins that participate in the mobilization of receptors to endocytotic organelles or to execute this mobilization themselves. Thus, β-arrestins might serve a more general function in receptor-mediated endocytotic pathways. Although the conclusions obtained from protein-overexpression studies in heterologous cell systems should be interpreted with caution, the physiological relevance of the observations presented here would seem to be substantiated by the documentation of both gain and loss of function with respect to the same paradigm, agonist-promoted GPCR sequestration.

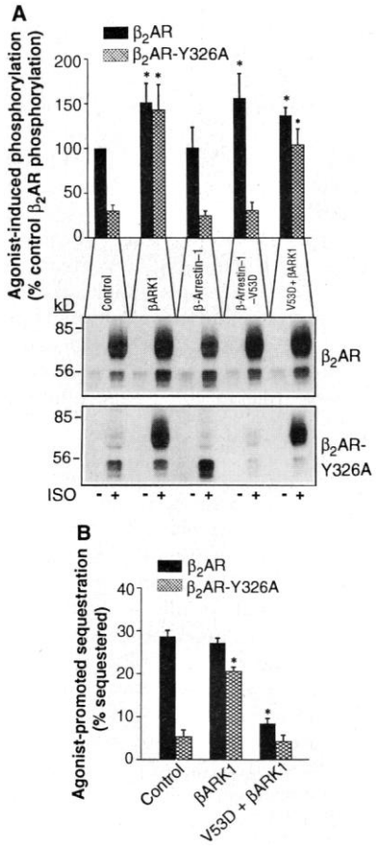

Fig. 4. Rescue of β_2AR-Y326A phosphorylation by βARK in the presence of β-arrestin–1–V53D, without the rescue of sequestration. (**A**) Representative autoradiographs for the whole-cell (HEK 293) phosphorylation (*22*) of β_2AR and β_2AR-Y326A after transient transfection with empty pcDNA1-Amp vector (control), 5 μg of pcDNA1 βARK1, 5 μg of pcMV5 β-arrestin–1, 10 μg of pcDNA1-Amp β-arrestin–1–V53D, or 5 μg of pcDNA1 βARK1 in tandem with 10 μg of β-arrestin–1–V53D (V53D + βARK1). Agonist-induced control β_2AR phosphorylation of the major β_2AR species (a glycoprotein ranging from 56 to 85 kD) was increased 4.6 ± 0.9–fold above basal. The mean ± SD for the quantitative analysis of four different experiments is illustrated in the bar graph above. (**B**) Agonist-promoted sequestration of β_2AR and β_2AR-Y326A receptor after transient transfection with empty pcDNA1-Amp vector (control), 5 μg of pcDNA1 βARK1 (βARK1), or 5 μg of pcDNA1 βARK1 with 10 μg of pcDNA1-Amp β-arrestin–1–V53D (V53D + βARK1). β_2AR and β_2AR-Y326A expression levels were 1775 ± 162 and 1570 ± 181 fmol/mg whole-cell protein, respectively, for the whole-cell phosphorylation experiments and 1444 ± 133 and 700 ± 66 fmol/mg protein, respectively, for the sequestration experiments. *$P < 0.05$ compared with matched control.

Our results suggest that by serving as molecular intermediates, β-arrestin proteins play a role in agonist-promoted β_2AR internalization. In addition, they demonstrate the role of βARK phosphorylation in β_2AR sequestration as facilitating the interaction of β-arrestin proteins with the receptor. The suggestion that β-arrestins serve as adaptor-like proteins indicates that βARK phosphorylation and β-arrestin binding to βARK-phosphorylated receptor represent early steps in the sequestration process. Our studies indicate that β-arrestins have dual functions in regulating β_2AR activity: not only do they bind and uncouple the receptor (*2*, *4*), but they participate in mediating receptor sequestration and consequently are likely to play a role in both the desensitization and the subsequent resensitization of the responsiveness of β_2AR and perhaps other GPCRs.

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14. The T^{341} truncation mutants of both wild-type and Y326A-β_2ARs were generated by polymerase chain reaction (PCR) to mutate codon 342 CTG (Leu) to TAG (stop). Positive clones were isolated for each mutant, and the integrity of the coding sequences as well as the mutation was confirmed by dideoxy DNA sequencing.
15. A point mutation in rat β-arrestin–1 ($V^{53} \rightarrow$ D) was generated by PCR to mutate codon GTC (valine) to GAC (aspartic acid). A point mutation in rat β-arrestin–2 ($V^{54} \rightarrow$ D) was generated by PCR to mutate codon GTG (valine) to GAT (aspartic acid). Positive clones were isolated for each mutant, and the integrity of the coding sequences as well as the mutation was confirmed by dideoxy DNA sequencing.
16. When tested, β-arrestin–1–V53D overexpression had no effect on the ability of the wild-type β_2AR to desensitize in HEK 293 cells (S. S. G. Ferguson *et al.*, data not shown).
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18. βARK1 protein was overexpressed greater than 20-fold above endogenous levels (*7*), and β-arrestin–1 and –2 proteins were overexpressed greater than 30-fold above endogenous levels as measured by densitometric analysis of immunoblots (S. S. G. Ferguson *et al.*, data not shown).
19. In brief, receptor sequestration after a 30-min exposure to agonist, 10 μM isoproterenol (ISO), was defined as the fraction of specific ^{125}I-pindolol (1 nM) binding performed at 14°C for 3 hours (*3*), which because of hydrophobicity can measure both surface and intracellular receptors not competed for by CGP-12177 (a hydrophilic ligand) minus the basal level of sequestered receptors as measured without exposure to agonist. Basally internalized receptors represented 28.5 ± 2% of total cell receptors. Overexpression of βARK1, β-arrestin–1, β-arrestin–2, β-arrestin–1–V53D, and β-arrestin–2–V54D had no effect on the number of basally internalized receptors.
20. Human embryonic kidney cells (HEK 293 cells) were grown in minimal essential medium with Earle's salts, supplemented with heat-inactivated fetal bovine serum (10% v/v) and gentamicin (100 μg/ml). The cells were seeded at a density of ~2.5 × 10^6 cells per 100-mm dish and transiently transfected with a modified calcium phosphate method [B. R. Cullen, *Methods Enzymol.* **152**, 684 (1987)].
21. β_2AR expression binding studies were done at 30°C for 30 min for the measurement of receptor expression, and bound ligand was separated on glass fiber filters (Whatman, GF/C) by vacuum filtration. The filters were washed four times with 4 ml of cold wash buffer [50 mM Tris and 120 mM NaCl (pH 7.2)] and counted in a gamma-counter. Protein concentrations were determined with a Bio-Rad assay kit with bovine serum albumin as the standard.
22. HEK 293 cells were labeled for 45 min at 37°C with 0.5 ml of [^{32}P]orthophosphate (100 μCi/ml) per well in serum- and phosphate-free Dulbecco's modified Ea-

gle's medium (DMEM), then treated with serum- and phosphate-free DMEM containing 100 μM ascorbate with or without ISO (10 μM final concentration) and incubated at 37°C for 15 min. The cells were then assayed for whole-cell phosphorylation as described (*7*). The extent of receptor phosphorylation was quantitated with a Molecular Dynamics phosphorimaging system and ImageQuant software.

23. We thank R.J. Lefkowitz for encouragement and insightful reading of the manuscript and members of his laboratory for antisera to βARK1 and -2, antisera to β-arrestin–1 and –2, wild-type βARK1, β-arrestin–1 and –2 cDNA constructs, and wild-type and phosphorylation site–deficient β_2AR cDNA constructs. Supported in part by a grant from the National Institutes of Health (NS 19576) and a Bristol Myers Squibb unrestricted grant award to M.G.C. S.S.G.F. is a fellow of the Medical Research Council of Canada, W.E.D. III is a recipient of a Howard Hughes Medical Institute research training fellowship for medical students, and L.S.B. is a recipient of a Howard Hughes postdoctoral fellowship.

3 October 1995; accepted 20 November 1995

The Effect of Social Experience on Serotonergic Modulation of the Escape Circuit of Crayfish

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The neuromodulator serotonin has widespread effects in the nervous systems of many animals, often influencing aggression and dominance status. In crayfish, the effect of serotonin on the neural circuit for tailflip escape behavior was found to depend on the animal's social experience. Serotonin reversibly enhanced the response to sensory stimuli of the lateral giant (LG) tailflip command neuron in socially dominant crayfish, reversibly inhibited it in subordinate animals, and persistently enhanced it in socially isolated crayfish. Serotonin receptor agonists had opposing effects: A vertebrate serotonin type 1 receptor agonist inhibited the LG neurons in dominant and subordinate crayfish and had no effect in isolates, whereas a vertebrate serotonin type 2 receptor agonist enhanced the LG neurons' responses in all three types of crayfish. The LG neurons appear to have at least two populations of serotonin receptors that differ in efficacy in dominant, subordinate, and socially isolate crayfish.

Serotonin is a neuromodulator involved in the expression of dominance and aggression in many animals, including humans (*1*). In lobsters and crayfish, serotonin injected into the circulatory system causes them to adopt an elevated, flexed ("dominant") posture (*2*). Serotonin-containing neurons function as postural gain-setting elements, biased toward flexion, that enhance the responsiveness of the motor circuitry to coordinated postural commands (*3*).

Serotonin plays a similar gain-setting role in the neural circuit for tailflip escape in crayfish (*4*); however, we found that the modulatory effect of serotonin depends on the social experience of the animal. The LG neuron is a command neuron for tailflip that is excited by mechanosensory input from the abdomen (Fig. 1) (*5*). It has been shown that serotonin applied through the arterial blood supply inhibits both the monosynaptic (α) and disynaptic (β) components of the compound excitatory postsynaptic potential (EPSP) evoked in the LG neuron by sensory nerve stimulation (*4*, *6*). Serotonin also depolarized the LG neuron by 2 to 3 mV and caused a drop in the input resistance of the neuron's distal dendrites, suggesting that serotonin's effects were mediated on the LG neuron itself. Serotonin-containing neurons and fibers with varicose terminals are present in each abdominal ganglion, but the relation between LG dendrites and the terminals of serotonergic neurons has not been described (*7*). Endogenous serotonin could affect the LG neuron's response through local ganglionic release or through neurohumoral release mechanisms.

When placed together in a small aquarium, a pair of previously isolated crayfish of similar size will interact agonistically to determine which animal is dominant and which is subordinate. The interactions usually take less than 0.5 hour, after which the subordinate moves by retreating or tailflipping to avoid contact with the dominant animal. We used these easily recognizable behavior patterns to determine the dominance status of animals paired for periods of 12 days or longer (*8*). We then determined the effect of bath-applied serotonin on the LG neuron's response to sensory nerve stimulation in socially isolated animals and in animals of known dominance status (*9*). In socially isolated animals, serotonin (50 and 100 μM) enhanced the LG neuron's responses to sensory nerve shock (Fig. 2A, left). The α and β EPSPs evoked in the LG neuron by all subthreshold levels of sensory nerve shock were increased, and the stimulus threshold was reduced (*10*). These effects were not readily reversible and persisted after 5 hours of wash. In subordinate crayfish, however, serotonin reversibly reduced the LG neuron's EPSPs (Fig. 2A, middle): α and β EPSPs evoked by all subthreshold levels of sensory nerve shock were reduced in the presence of bath-applied serotonin, and the stimulus threshold of an LG spike was increased (*10*). A 1-hour wash with saline restored the LG EPSPs and in some instances produced a rebound excitation. In dominant crayfish, serotonin reversibly enhanced LG responses over the complete range of subthreshold stimuli (Fig. 2A, right), and reduced the LG neuron's stimulus threshold (*10*). We obtained similar results in 37 juvenile (Fig. 3A) and 23 adult (*10*) crayfish. Serotonin had no obvious effect on the responses of other mechanosensory interneurons, some of which contribute to the β EPSP in the LG neuron (Fig. 1) (*11*). We conclude that serotonin's modulatory effect on the LG neuron's response depends on the animal's social experience.

To determine if the different effects of serotonin on the LG neuron in dominant, subordinate, and socially isolated crayfish were produced by different serotonin receptors, we repeated the experiments described above with a vertebrate serotonin type 1 (5-HT_1) or serotonin type 2 (5-HT_2) receptor agonist substituted for serotonin. Although the vertebrate 5-HT_1 agonist 1-(3-chlorophenyl)piperazine dihydrochloride (m-CPP Cl_2) had no effect on the LG neuron's responses in the social isolates, it reduced the α and β LG EPSPs evoked by the entire range of subthreshold stimuli in all dominant and subordinate crayfish (Fig. 2B and Fig. 3B), and it raised the stimulus

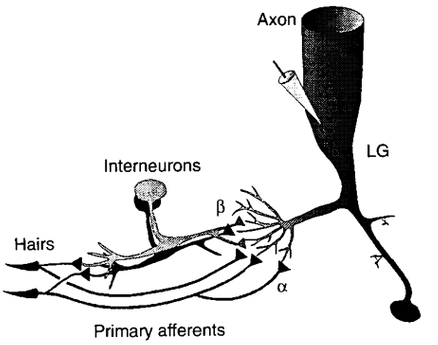

Fig. 1. Afferent portion of the LG tailflip circuit, showing monosynaptic (α) and disynaptic (β) afferent paths from abdominal hairs to the LG neuron (*5*). A single spike in the LG is sufficient to trigger tailflip escape movements. Afferent nerves from the sensory hairs were stimulated in the presence of serotonin and 5-HT agonists; LG EPSPs were recorded from the proximal axon (*9*).

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