Effects of Restraint Stress on Components of Adenylyl Cyclase Signal Transduction in the Rat Hippocampus

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Chronic stress can injure hippocampal neurons as well as alter hippocampal function. The adenylyl cyclase (AC) signal transduction system is an important modulator of neurotransmission in this brain region. This study was conducted to begin to understand the effects of chronic stress on the hippocampal AC system. To assess dependence of type I and type II AC mRNA expression on adrenal integrity, total RNA was prepared from the hippocampus of nonstressed rats 7 days following either sham surgery or adrenalectomy (ADX). Adrenalectomy resulted in a 60% diminution in steady-state expression of type II AC mRNA ($p < .005$) and a nonsignificant fall in type I AC mRNA expression. Both sham and adrenalectomized rats were then exposed to 1-hour sessions of restraint, twice per day (0800 to 0900 and 1500 to 1600 hours) for 4 days. Following the stress paradigm, RNA was prepared from the hippocampus and type I and type II AC mRNA levels determined by Northern blot. Densitometic analysis showed that in comparison to unstressed sham rats, stressed sham rats had a significant twofold increase in steady-state levels of type I and type II mRNA. Stressed adrenalectomized rats also had increased expression in type II AC mRNA but no significant stress-induced change in expression of type I AC mRNA. To assess dependence of hippocampal membrane G-Proteins on adrenal integrity, hippocampal membranes from nonstressed rats 7 days following either sham surgery or adrenalectomy (ADX) were analyzed by immunoblot. Adrenalectomy resulted in a 25% diminution in membrane Gsα content ($p < .05$), but ADX did not significantly change membrane Gi(1)α, Gi(2)α, $G\beta_{36}$ or Goα content. In Sham animals, the stress paradigm resulted in increased levels of membrane Gsα, Goα and Gβ. Adrenalectomy blocked the effects of stress on these G-protein changes. Restraint stress did not modulate the levels of Gi(1)α or Gi(2)α. Restraint stress induced a small but significant increase in both AC activity and cAMP levels in hippocampus of both sham and adrenalectomized rats. In summary, components of the hippocampal AC signal transduction system and Goα are modulated by factors released during stress.
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KEY WORDS: *Chronic stress; Hippocampus; Hippocampal AC signal transduction system*

Stress constitutes any perturbation of homeostasis. The release of adrenal glucocorticoids and catecholamines under conditions that threaten homeostasis represents one of the central adaptive mechanisms among mammals. Adrenal-derived stress hormones increase free fatty acid levels, elevate blood glucose, and assist the organism by increasing the availability of energy substrates (Munck et al. 1984). Furthermore, glucocorticoids play a key role in regulating cytokine production and immune function (Sternberg et al. 1989). There is great individual variability in the magnitude and duration of the stress response (Sternberg et al. 1989). There is growing appreciation that excessive production of stress hormones during chronic stress can have deleterious consequences on biological fitness (Meaney et al. 1988; Sternberg et al. 1989; Sapolsky and Plotsky, 1990).

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Mechanisms underlying stress-induced CNS injury have not been well characterized. However, recent evidence shows that chronic stress can modulate components of adenylyl cyclase (AC) signal transduction in the pituitary and influence important cellular function (Morrill et al. 1993).

The AC complex is composed of three separate proteins embedded in the plasma membrane: receptors, GTP-binding proteins (G-proteins), and catalytic protein (AC) (Gilman 1990). G proteins are heterotrimers, consisting of α, β, and γ subunits. The α subunits, which contain the guanine nucleotide binding site and GTPase activity, vary among G-proteins and confer on them their specificity of interaction with receptors and effectors. Two G proteins, Gs and Gi, mediate hormone and/or neurotransmitter stimulation or inhibition, respectively, of adenylate cyclase activity. There are four forms of Gsα, two large (52 kD) and two small (45 kD), derived from alternative splicing of a single Gsα mRNA (Van Sande et al. 1990; Chabre et al. 1992;). Three different Gi proteins (arbitrarily designated Gi_1, Gi_2, and Gi_3) have been identified and found to be independent gene products (Roff et al. 1985; Mumby et al. 1986). Inhibition of AC in platelets by α_2-adrenergic agonists can be blocked by pretreating platelet membranes with an antibody to αi_2, thus implicating Gi_2 in the inhibition of adenylate cyclase (Taussig et al. 1993). Gi_3 may be a modulatory of potassium channel activity (Codina et al. 1986).

The βγ dimer is tightly associated with a GDP-bound α chain and facilitates interaction of the G protein with a receptor molecule. In addition to their role regulating α chain function, the βγ subunits themselves appear capable of transmitting intracellular messages in some cells and may anchor the αβγ complex to the cell membrane (Tang and Gilman 1991).

Finally, there are multiple forms of AC, each a unique gene product (Mumby et al. 1986; Feinstein et al. 1991; Gao and Gilman 1991; Tang et al. 1991; Tang and Gilman 1992). The brain contains a calmodulin sensitive (Type I) and a calmodulin-insensitive (Type II) AC as well as an AC found in olfactory tissue (Type III) and in striatum (Type IV).

In this study, we investigated the effects of restraint stress on the various components of the AC system in the hippocampus of the rat.

METHODS

Restraint Stress

Male Fisher/344 rats (Harlan Sprague-Dawley; Indianapolis, IN) weighing 200 to 250 g were housed five per cage in a quiet temperature- (22°C) and light-controlled environment, (12 hours of light, 12 hours of darkness). Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Seven days prior to stress experiments, rats underwent sham surgery or adrenalectomy (Harlan) via 10 mm bilateral flank incisions following intramuscular injection of Ketamine (4.1 mg) with Xylazine (0.85 mg/100 gm body weight); adrenalectomized rats were maintained on 0.9% saline. There was mortality in either group. Rats were randomized to control or stress groups. Rats were exposed to 1-hour sessions of restraint, twice per day (0800 to 0900 hours and 1500 to 1600 hours) for 4 days (Hauger et al. 1990). Control rats were left in their cages. At the completion of stress, rats were quickly guillotined, indicated tissues removed, and blood immediately collected. Serum was prepared and stored at −70°C. Plasma corticosterone was measured by RIA (ICN, Bakersfield, CA).

Membrane Preparation

Membranes were prepared as described (Wand et al. 1993). Tissue homogenates were spun at 500 × g for 5 minutes at 4°C to remove the nuclear pellet. The supernatant was then spun at 20,000 × g for 20 minutes at 4°C. The pellet was washed twice in wash buffer (lysis buffer without sucrose). The final membrane pellet was diluted with wash buffer and stored at −70°C. Protein concentration was determined by bicinchonic acid reaction with BSA as standard.

Adenylyl Cyclase Activity

Adenylyl cyclase activity of membrane preparations was determined in triplicate by a modification of the method of Salomon et al. (1974). Aliquots (5 to 20 μg) of membrane protein were assayed in 100 μl final volume containing 0.1 mmol/Lm (α-^{32}P)-ATP (1 μCi), 2.8 U creatine phosphokinase, 5 mmol/L creatine phosphate, 1.5 mmol/L $MgCl_2$, 19.2 mg% BSA, 50 mmol/L Na-Hepes (pH 7.6), 0.3 mmol/L KCL, 0.2 mmol/L cAMP, and 1 mmol/L dithiothreitol. Samples were incubated at 30°C for 20 minutes with no further additions (basal) or in the presence of 10 mmol/L NaF or 10 μmol/L GTP-γ-S. The reaction was terminated by the addition of 100 μl 50 mmol/L Hepes (pH 7.5), 2 mmol/L ATP, 0.5 mmol/L 3H- cAMP (15 nCi), 2% SDS, and heating to 100°C for 3 minutes. Cyclic AMP was isolated by chromatography. Recovery averaged 87%. Cyclic AMP levels were measured from hippocampal acid extracts as previously described (Wand et al. 1993).

Immunoblot Analysis

For immunoblot analysis (Wand et al. 1993), membrane proteins (50 μg) were fractionated by electrophoresis through 10% SDS-polyacrylamide gels (10% acryl-

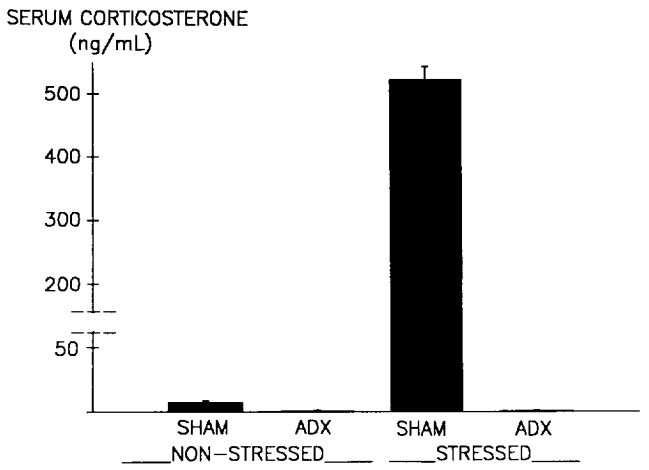

Figure 1. Effects of ADX and restraint stress on serum corticosterone levels. Serum corticosterone levels were determined in nonstressed/sham (n = 8), nonstressed/ADX (n = 7), stressed/sham (n = 8), and stressed/ADX (n = 8) groups of rats.

amide, 0.13% phenylpiperazine). Proteins were electrophoretically transferred to polyvinylidine difluoride filters using a transfer bath containing 10% methanol, 0.01 mol/L 3-(cyclohexlamino)-1-Propanesulfonic acid, pH 11.0). After transfer, membranes (filters) were stained with Coomassie blue to ascertain transfer efficacy. Filters were incubated for 2 hours at room temperature in 50 mmol/L Tris, 138 mmol/L NaCl, 2 mmol/L $MgCl_2$, pH 7.4 T(TBS) containing 3% BSA, 0.1% Tween-20, .02% NaN3 and washed twice for 5 to 10 minutes with TBS containing 0.2% SDS, 2% Nonidet P-40. Filters were incubated overnight at room temperature with specific primary antibodies in TBS containing 1% BSA, 0.05% Tween-20, 0.02% NaN3, and 2% Nonidet P-40. Filters were washed twice for 30 minutes in wash buffer (TBS, 0.2% SDS, 2% Nonidet P-40) and incubated for 2 hours with ^{125}I-protein A (0.5 μCi/ml) in 1% BSA, 0.05% Tween-20 0.02% NaN_3, and 2% Nonidet P-40. Filters were washed twice for 30 minutes and then rinsed twice for 5 to 10 minutes in wash buffer and autoradiographed. Autoradiographic image intensities were determined by two dimensional densitometry using the Molecular Dynamics personal densitometer system. Antiserum C584 recognizes C-terminal amino acids (325 to 339) of both the 45- and 52-kD forms of Gsα (Wand et al. 1993). Antiserum A54 recognizes Gi(2)α, a 41-kD protein (Wand et al. 1993). Antiserum A56 recognizes Gi(1)α and Gi(3)α (Wand et al. 1993). Antisera GC4 recognizes Gβ36 and Gβ35 (Wand et al. 1993).

RNA Analysis

RNA was extracted by the guanidium isothiocyanate method and isolated by centrifugation through a 5.7 mol/L cesium chloride cushion (Chirgwin et al. 1979). RNA OD 260/280 ratios ranged between 1.9 and 2.2. Total RNA was size-fractionated on 0.8% formaldehyde-agarose gels. Integrity of the RNA preparation was assessed by ethidium bromide staining and UV (254 nm) illumination. Resolved RNA was transferred to Gene Screen Plus® nylon membranes and prehybridized and hybridized as described using random primer labeled type I and type II AC cDNA probes. Autoradiographic image intensities were determined using the Molecular Dynamics personal densitometer system. Specific mRNA signals were normalized to the hybridization signal of 18s RNA or actin mRNA.

Statistics

Differences between groups and individuals were evaluated by the Student t-test where appropriate.

RESULTS

To assess dependence of steady-state levels of type I and type II adenylyl cyclase (AC) mRNA on adrenal function, total RNA was prepared from the hip-

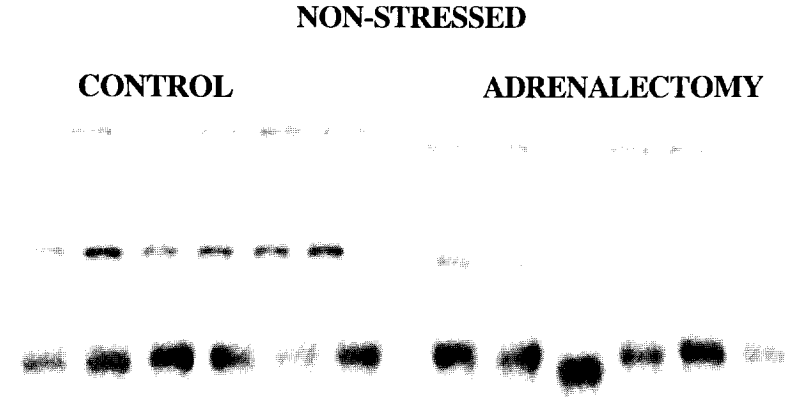

Figure 2. Representative Northern demonstrating adrenal dependence of steady-state levels of type I and II AC mRNA in the hippocampus. Seven days following sham surgery or adrenalectomy (ADX), steady-state expression of hippocampal type I and type II AC mRNA levels were assessed in nonstressed rats. Data are derived from individual hippocampi, and RNA was not pooled.

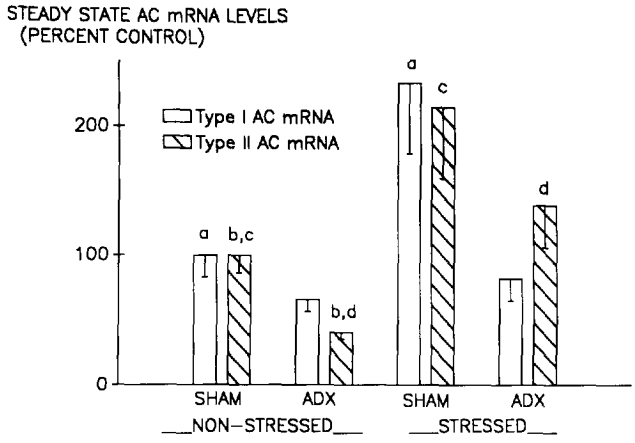

Figure 3. Densitometric analysis of the effects of restraint stress on steady state levels of type I and type II AC mRNA in the hippocampus. a, $p < .05$; b, $p < .005$; c, $p < .05$; d, $p < .02$.

pocampus of nonstressed rats 7 days following sham surgery of adrenalectomy (ADX). Adrenalectomy resulted in undetectable serum corticosterone levels (Figure 1). The Type I AC cDNA probe recognized an 11-kb mRNA, whereas the type II AC cDNA probe recognized a 4.1-kb mRNA. A representative Northern blot demonstrates that ADX resulted in a fall in steady-state expression of type I and type II AC mRNA (Figure 2). Densitometric analysis confirmed that ADX resulted in 60% fall in type II AC mRNA levels ($p < .02$), and a nonsignificant drop in the expression of type I AC mRNA (Figure 3). Sham and adrenalectomized rats were then exposed to 1-hour sessions of restraint, twice per day (0800 to 0900 and 1500 to 1600) for 4 days. Nonstressed (Control) rats were left in their cages. At the completion of stress, rats were immediately guillotined, hippocampal tissue removed, trunk blood collected, and serum prepared. Following the final stress period, serum corticosterone levels were significantly elevated in the sham stressed group (Figure 1).

Following the stress paradigm, RNA was prepared from hippocampal tissue and type I and type II AC mRNA levels determined by Northern blot. Densitometric analysis of audoradiographs derived from Northern blots (Figure 3) show that stressed sham rats had a significant two-fold increase in both type I and type II AC mRNA following stress compared to nonstressed sham rats ($p < .05$). Stressed adrenalectomized rats also showed increased expression in type II AC mRNA (Figure 3) compared to expression of type II AC mRNA in nonstressed adrenalectomized rats ($p < .02$). Although restraint stress increased expression of type II AC mRNA in both sham and adrenalectomized rats in comparison to their nonstressed counterparts, expression of the type II mRNA was higher in the sham group ($p < .05$). In contrast, restraint stress did not increase expression of type I AC mRNA in adrenalectomized rats.

To assess dependence of hippocampal membrane G-Proteins on adrenal integrity, hippocampal membranes from nonstressed rats 7 days following either sham surgery of adrenalectomy (ADX) were analyzed by immunoblot. Compared to Sham, ADX resulted in a 25% diminution in membrane Gsα content ($p < .05$), but ADX did not significantly change membrane Gi(1)α, Gi(2)α, $G\beta_{36}$, or Goα content (Table 1). In Sham animals, the stress paradigm resulted in increased levels of membrane Gsα, Goα, and Gβ (Table 1). However, adrenalectomy blocked the effects of stress on these G-protein changes (Table 1). Although restraint stress did not alter the levels of Gi(3)α and Gi(2)α in sham animals, there was a 40% reduction in membrane Gi(1)α content in ADX rats exposed to stress.

Hippocampal membrane AC activity as well as cAMP content was determined to assess the functional consequences of ADX and restraint stress on AC mRNAs and G-protein modulation. In the nonstress setting, ADX resulted in a 25% reduction in hippocampal membrane AC activity as well as in cAMP concentrations compared

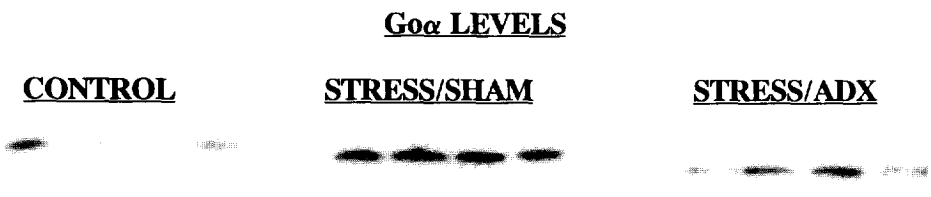

Figure 4. Representative immunoblot of Goα levels of the hippocampus. Nonstressed sham ($n = 8$), stressed/sham ($n = 8$), and stressed/ADX ($n = 8$) groups of rats were stressed as described in Methods. Following the final stress session, rats were sacrificed, hippocampal membranes prepared, and immunoblots performed as described in Methods. Data are derived from individual hippocampi, and membranes were not pooled.

Table 1. Immunoblot Analysis

Paradigm	Gsα	Gi(2)α	Gi(1)α	Goα	Gβ36
Nonstressed/Sham	100 ± 9	100 ± 13	100 ± 5	100 ± 8	100 ± 6
Nonstressed/ADX	77 ± 7[a]	97 ± 5	106 ± 7	95 ± 7	87 ± 5
Stressed/Sham	124 ± 12[a]	114 ± 5	82 ± 14	137 ± 1[b]	130 ± 5[c]
Stressed/ADX	98 ± 15	104 ± 10	59 ± 6[b]	75 ± 20	119 ± 13

[a] $p < .05$.
[b] $p < .001$.
[c] $p < .005$.

Table 2. Hippocampal Adenylyl Cyclase Activity and cAMP Concentration

	Adenylyl Cyclase Activity (*pmol/mg protein*)			cAMP Concentration	
	NaF	GTPγS	*n*	(*pmol/mg protein*)	*n*
Nonstressed/Sham	2306 ± 56	957 ± 52	6	23 ± 2.4	6
Nonstressed/ADX	1524 ± 50[a]	655 ± 88[c]	6	20 ± 2.5	7
Stressed/Sham	2689 ± 70[a]	1054 ± 33	8	31 ± 1.8[b]	6
Stessed/ADX	2640 ± 109[b]	1184 ± 71[c]	8	40 ± 3.3[a]	8

[a] $p < .005$.
[b] $p < .025$.
[c] $p < .05$.

to sham condition (Table 2). Restraint stress induced a small but significant increase in both AC activity and cAMP levels in hippocampus of both sham and adrenalectomized rats (Table 2).

DISCUSSION

Adenylyl cyclase catalyzes the formation of cAMP, an important intracellular second messenger in many eukaryotic cells including neurons of the central nervous system (Krebs and Beavo 1979; Cheung and Storm 1982; Nairn et al. 1985; Levitzki 1987; Greenberg et al. 1993). In the present study, we show that stress can modulate expression of the stimulatory arm of the adenylyl cyclase signal transduction system as well as expression of Goα in the rat hippocampus. Restraint stress was associated with enhanced expression of type I and type II adenylyl cyclase mRNA expression as well as being associated with enhanced expression of Gsα, Goα, and Gβ36. Adrenalectomy reduced expression of type II AC mRNA and Gsα in both unstressed and stressed rats compared to unstressed and stressed sham rats. The effects of stress on type I AC mRNA expression were not present if rats were adrenalectomized prior to the stress protocol. The effects of stress on type II AC mRNA expression were attenuated if rats were adrenalectomized. In a similar manner, stress did not enhance expression of Gsα, Goα, and Gβ36 in the adrenalectomized rats. This suggests that glucocorticoids or catecholamines are in part responsible for maintaining expression of type I and type II adenylyl cyclase mRNA in the nonstressed state and also responsible for increasing expression of both messages and G-proteins during restraint stress. Expression of Gi(1)α or Gi(2)α were not altered by stress.

Both membrane adenylyl cyclase activity and hippocampal cAMP content was increased during restraint stress, even in stressed adrenalectomized rats. The maintenance of cAMP levels in ADX animals during stress may be the result of a significant fall in levels of the inhibitory G-protein, Gi(1)α. At this time, we do not know if enhanced expression of AC mRNA was paralleled by an increase in AC membranes protein. Likewise, we do not know if changes in G-protein expression are due to transcriptional or post-transcriptional events.

Because total adrenalectomy was performed (medulla and cortex), the data do not allow us to determine whether adrenal-derived catecholamines or glucocorticoids are responsible for the stress-induced regulation of type I and type II adenylyl cyclase mRNA. However, there are several precedents that have established a role for glucocorticoids in the regulation of AC signal transduction. For example, glucocorticoids increase expression of Gsα in GH3 cells, a rat pituitary cell line (Chang and Bourne 1987). The mechanism by which glucocorticoids increase expression of Gsα is unknown, but the effect is blocked by protein synthesis inhibitors (Chang and Bourne 1987). In rats, 7-day exposure to high levels of glucocorticoids will increase Gsα levels in the frontal cortex (Saito et al. 1989). Adipocyte Gsα levels fall

following adrenalectomy and normalize again with glucocorticoid replacement (Ros et al. 1989). Moreover, glucocorticoids increase adenylyl cyclase activity in astrocytoma cells and also in vascular smooth muscle (Balmforth et al. 1989; Yasunari et al. 1989). Recently, we showed that stress increases expression of type II adenylyl cyclase mRNA and adenylyl cyclase activity in the rat pituitary (Morrill et al. 1993). Both morphine and cocaine have been shown to modulate the expression of Goα (Nestler et al. 1989; Colin et al. 1991).

There is much evidence for deleterious consequences of glucocorticoids and stress. Excess production of endogenously generated corticosterone results in loss of hippocampal type II glucocorticoid receptors, neuronal injury, and hippocampal-mediated neurological deficits (Landfield et al. 1978; Salpolsky et al. 1984, 1985; Meaney et al. 1991). Animals treated for 3 months with exogenous corticosterone in the upper physiological range (mimicking the elevated basal levels seen in certain rats) show profound hippocampal cell loss (CA_1 and CA_3 cell fields) (Salpolsky et al. 1984). In the rat, a reduction in total lifetime glucocorticoid exposure prevents some of the neuronal loss and spacial learning deficits that accompany aging.

In mammalian brain, adenylyl cyclase activity is regulated by neurotransmitter and hormone receptors that are coupled to the enzyme through the G regulatory proteins, Gs and Gi (Nestler and Greengard 1983). Protein phosphorylation catalyzed by cAMP-dependent protein kinase A regulates several important aspects of neuronal function, including ion channel activity, gene expression, and neurotransmitter synthesis (Nairn et al. 1985; Greenberg et al. 1993). Furthermore, cAMP has been implicated in the regulation of synaptic plasticity and may play an important role in mechanisms underlying learning and memory (Kandel and Schwartz 1982; Dudai and Zvi 1984; Dudai 1988). We speculate that modulation of adenylyl cyclase activity contributes to altered hippocampal function following chronic stress. In man, such observations may explain the pathological consequences of certain disease states associated with excess cortisol production.

We conclude that the stimulatory arm of the adenylyl cyclase signal transduction system as well as Goα are targets of factors released during stress. We speculate that chronic stress has deleterious effects on hippocampal adenylyl cyclase signal transduction, which may impact on health and disease over the lifetime of the individual.

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