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Chronic Caffeine Alters the Density of Adenosine, Adrenergic, Cholinergic, GABA, and Serotonin Receptors and Calcium Channels in Mouse Brain

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SUMMARY

1. Chronic ingestion of caffeine by male NIH strain mice alters the density of a variety of central receptors.

2. The density of cortical A_1 adenosine receptors is increased by 20%, while the density of striatal A_{2A} adenosine receptors is unaltered.

3. The densities of cortical β_1 and cerebellar β_2 adrenergic receptors are reduced by *ca.* 25%, while the densities of cortical α_1 and α_2 adrenergic receptors are not significantly altered. Densities of striatal D_1 and D_2 dopaminergic receptors are unaltered. The densities of cortical 5 HT_1 and 5 HT_2 serotonergic receptors are increased by 26–30%. Densities of cortical muscarinic and nicotinic receptors are increased by 40–50%. The density of cortical benzodiazepine-binding sites associated with $GABA_A$ receptors is increased by 65%, and the affinity appears slightly decreased. The density of cortical MK-801 sites associated with NMDA-glutaminergic receptors appear unaltered.

4. The density of cortical nitrendipine-binding sites associated with calcium channels is increased by 18%.

5. The results indicate that chronic ingestion of caffeine equivalent to about 100 mg/kg/day in mice causes a wide range of biochemical alterations in the central nervous system.

Keywords

caffeine; adenosine receptors; adrenergic receptors; cholinergic receptors; serotonin receptors; GABA receptors; calcium channels; dopamine receptors; NMDA receptors

INTRODUCTION

Chronic caffeine can cause tolerance in animals and humans, and subsequent abstinence from caffeine can then lead to withdrawal syndromes. The underlying mechanisms are poorly understood (Nehlig *et al.,* 1992; Daly, 1993). Blockade of central adenosine receptors resulting in an up-regulation of adenosine receptors has received attention as one explanation of the chronic effects of caffeine. Chronic treatment with caffeine does result in an apparent up-regulation of A_1 adenosine receptors in rodents (Fredholm, 1982; Boulenger *et al.,* 1983; Wu and Coffin, 1984; Green and Stiles, 1986; Zielke and Zielke, 1987; Hawkins *et al.,* 1988; Ramkumar *et al.,* 1988; Daval *et al.,* 1989; Rudolphi *et al.,* 1989; see also references in Nehlig *et al.,* 1992; Daly, 1993), and an enhanced sensitivity to behavioral effects of adenosine analogs (Ahlijanian *et al.,* 1986; Nikodijević *et al.,* 1993). The effects of chronic caffeine on other central receptors has received less attention. β-Adrenergic

receptors appear down-regulated, while α-adrenergic receptors appear unchanged (Goldberg *et al.,* 1982; Green and Stiles, 1986). Caffeine does alter turnover of not only norepinephrine, but also of dopamine and serotonin (Berkowitz *et al.,* 1970; Berkowitz and Spector, 1971; Corrodi *et al.,* 1972; Hadfield and Milio, 1989). The effects of chronic caffeine on levels of dopamine and serotonin receptors do not appear to have been assessed. Similarly, while behavioral effects of cholinergic agonists and antagonists are altered after chronic caffeine (Nikodijević *et al.,* 1992), changes in muscarinic and nicotinic receptors have not been assessed. The density of benzodiazepine sites associated with $GABA_A$-receptors appear increased after chronic caffeine (Wu and Coffin, 1984; Wu and Phillis, 1986).

The effect of chronic ingestion of caffeine on a variety of central receptors and on calcium channels has now been probed using radio ligand binding assays with male NIH-Swiss strain mice. The results provide evidence for significant alterations in adenosine, adrenergic, serotonergic, cholinergic and GABAergic systems. Such changes may underlie the changes in behavioral responses of such mice seen after chronic caffeine (Nikodijević *et al.,* 1993).

METHODS

Materials

[^{3}H] CGS21680 (specific activity, 47.2 Ci/mmol), [^{3}H]N^6 cyclohexyladenosine (30.2 Ci/mmol), [^{3}H] dihydroalprenolol (57·5 Ci/mmol), [^{3}H] SCH 23390 (71.3 Ci/mmol), [^{3}H]nicotine (75 Ci/mmol), [^{3}H]quinuclidinylbenzilate (45.5 Ci/mmol), [^{3}H]nitrendipine (71.2 Ci/mmol), [^{3}H]diazepam (84.5 Ci/mmol), [^{3}H]5-hydroxytryptamine (24.4 Ci/mmol), [^{3}H]ketanserin (64.1 Ci/mmol), [^{3}H]clonidine (60.0 Ci/mmol), [^{3}H]prazosin (18.1 Ci/mmol), [^{3}H]MK-801 (22.5 Ci/mmol) and [^{3}H]spiperone (23.1 Ci/mmol) were from New England Nuclear (Boston, MA). Caffeine (free base) was from Matheson, Coleman and Bell (Cincinnati, OH). R-N^6-Phenylisopropyladenosine, 2-chloroadenosine, c1onidine, phentolamine, MK-801, nicotine, pargyline, and methysergide were from Biochemicals, Inc. (Natick, MA). Propranolol, diisopropylfluoro-phosphate, polyethylenimine, and butaclamol were from Sigma (St. Louis, MO). Atropine was from Merck & Co., Inc. (Rahway, NJ). Nifedipine was from Delbay Pharmaceuticals Inc. (Bloomfield, NJ). Serotonin was from Regis Chemical Co. (Chicago, IL). R015-1788 was from Hoffmann La Roche, Inc. , NJ).

Chronic Caffeine Administration

White male mice of the NIH Swiss strain, weight 25–30 g, were divided into two groups. The control group was given free access to water and food, while the chronic caffeine group was given access to water containing 0.1 % caffeine for 4 days and to food. Caffeine ingestion was continued up to two to four hours before sacrifice. Based on water consumption, mice ingested ~100 mg/kg of caffeine per day. For further details and behavioral studies with such mice see Nikodijević *et al.* (1992).

Membrane Preparation

The mice were killed by cervical fracture and the brains rapidly removed into ice-cold 50 mM Tris-HCl buffer (pH 7.4). Cerebral cortex (5 mice), cerebellum (4 mice) or striatum (10 mice) were dissected from mice of either control or caffeine groups. Tissue was homogenized in 3 ml of ice-cold 50 mM Tris-HCI buffer (pH 7.4), using a polytron at setting number 6 for 10 seconds. The homogenate was adjusted in volume to 7 ml using ice-cold 50 mM Tris-HCl (pH 7.4) and centrifuged at 35000 × g for 15 min at 4°C. The pellet was suspended with polytron and recentrifuged in the same volume of buffer. The final pellet was resuspended in the appropriate incubation buffer at protein concentration of 2 3 mg/ml. Aliquots were used immediately for receptor-binding assays. Protein concentrations

were determined by the BCA protein assay reagents (Pierce Chemical Co., Rockford, IL) using bovine albumin as standard.

[^{3}H]N^6-Cyclohexyladenosine (CHA) Binding

[^{3}H]CHA binding to cortical membranes was measured by a modification of the method of Bruns *et al.* (1980). The final pellet had been resuspended in 50 mM Tris-HCl buffer (pH 7.4), containing 8 μg/ml adenosine deaminase. Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]CHA from 0.5 16 nM. The final volume was 1 ml. Each assay (90 min, 25°C) was performed in triplicate. Nonspecific binding was defined in the presence of 10 μM 2-chloroadenosine and was *ca.* 5% of the total binding. Binding reactions were terminated by filtration through Whatman GF/B filters using a Brandel M24R Cell Harvester (Brandel, Gaithersburg, MD). Filters were washed two times with 5 ml ice-cold buffer and placed in scintillation vials with 5 ml of Hydrofluor scintillation fluid, followed by counting for tritium.

[^{3}H]CGS 21680 Binding

[^{3}H]CGS 21680 binding to striatal membranes was measured by a mOdification of the method of Jarvis *et al.* (1989). The final pellet had been resuspended in 50 mM Tris-HCl (pH 7.4) buffer containing 8 μg/ml adenosine deaminase and 10 mM $MgCl_2$. Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]CGS 21680 from 0.5–16 nM. The final volume was 1 ml. Each assay (90 min, 25°C was performed in triplicate. Nonspecific binding was defined in the presence of 30 μM N^6-R-phenylisopropyladenosine and was *ca.* 20% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]Prazosin Binding

[^{3}H]Prazosin binding to cortical membranes was measured by a modification of the method of Glossman and Hornung (1980). The final pellet had been suspended in 50 mM Tris HCl buffer (pH 7.4). Incubations were in the same buffer, containing 100 μl membrane suspension and [^{3}H]prazosin from 0.05–0.8 nM. The final volume was 1 ml. Each assay (30 min, 25°) was performed in triplicate. Nonspecific binding was defined in the presence of 1 μM phentolamine and was *ca.* 10% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]Clonidine Binding

[^{3}H]Clonidine binding to cortical membranes was measured by a modification of the method described by U'Prichard *et al.* (1977). The final pellet had been resuspended in 50 mM Tris HCl buffer (pH 7.4). Incubations were in the same buffer containing l00 μl membrane suspension and [^{3}H]clonidine from 0.25 16 nM. The final volume was 1 ml. Each assay (30 min, 25°C) was performed in triplicate. Nonspecific binding was defined in the presence of 10 μM clonidine and was *ca.* 10% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]Dihydroalprenolol (DHA) Binding

[^{3}H]DHA binding to cortical membranes for β_1 adrenergic receptors and to cerebellum membranes for β_2 adrenergic receptors was measured by a modification of the method of Bylund and Snyder (1976). The final pellet had been resuspended in 50 mM Tris HCl buffer (pH 7.4), containing 10 mM $MgCl_2$ and 1 mM EDTA. Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]DHA from 0.125–8 nM. The final volume was 1 ml. Each assay (30 min, 30°C) was performed in triplicate. Nonspecific binding was

defined in the presence of 10 μM propranolol and was *ca.* 30% of total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]SCH 23390 Binding

[^{3}H]SCH 23390 binding to the D_1 dopaminergic receptor in striatal membranes was measured essentially as described (Ferretti, *et al.,* 1992). The final pellet had been resuspended in 50 mM Tris HCl buffer (pH 7.4), containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]SCH 23390 from 0.05–3.2 nM. The final volume was 1 ml. Each assay (30 min, 37°C) was performed in triplicate. Nonspecific binding was defined in the presence of 10 μM butaclamol and was *ca.* 5% of total bmdmg. Filtration, washing and scintilation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]Spiperone Binding

[^{3}H]Spiperone binding to D_2-dopaminergic receptors in striatal membranes was measured essentially as described (Michaluk *et al.,* 1982). The final pellet had been resuspended in 50 mM Tris HCl buffer (pH 7.4), containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM pargyline, and 0.1% ascorbic acid. Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]spiperone from 0.05–3.2 nM. Each assay (25 min, 37°C) was performed in triplicate. Nonspecific binding was defined in the presence of 1 μM butaclamol and was *ca.* 10% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]Nicotine Binding

[^{3}H]Nicotine binding to cortical membranes was measured by a modification of described procedures (Martino Garrows and Kellar, 1986; Marks *et al.,* 1986). The final pellet had been resuspended in 20 mM Hepes buffer (pH 7.4), containing 1 mM $MgCl_2$, 120 nM NaCl, 5 mM KCl, and 2 mM $CaCl_2$. Incubations were in the same buffer containing 100 μl membrane suspension, 200 μM diisopropyl-fluorophosphate and [^{3}H]nicotine from 0.125–8.0 nM. The final volume was 1 ml. Each assay (120 min, 0–4°C) was performed in triplicate. Nonspecific binding was defined in the presence of 1 μM nicotine and was *ca.* 20% of total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA. The filters were presoaked in cold 0.3% polyethylenimine to reduce nonspecific binding.

[^{3}H]Quinuclidinylbeuzilate (QNB)

[^{3}H]QNB binding to muscarinic receptors in cortical membranes was measured as follows: (see Yamamura and Snyder, 1979; Hammer *et al.,* 1980). The final pellet had been resuspended in 20 mM Hepes buffer (pH 7.4), containing 100 mM NaCl and 10 mM $MgCl_2$. Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]QNB from 0.05–0.8 nM. The final volume was 1 ml. Each assay (30 min, 37°C) was performed in triplicate. Nonspecific binding was defined in the presence of 1 μM atropine and was *ca.* 25% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [3H]CHA. The filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding to filter.

[^{3}H]Diazepam Binding

[^{3}H]Diazepam binding to benzodiazepine sites on $GABA_A$ receptors in cortical membranes was measured essentially as described (Wu and Coffin, 1984). The final pellet had been resuspended in 50 mM Tris-HCl buffer (pH 7.4), Incubations were in the same buffer containing 100 μl membrane suspension and [^{3}H]diazepam from 0.5–16nM. The final

volume was 1 ml. Each assay (60 min, 0 4°C) was performed in triplicate. Nonspecific binding was defined in the presence of 20 μM Ro15-1788 and was *ca.* 10% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]5-Hydroxytryptamine Binding

[^{3}H]5-Hydroxytryptamine binding to 5-HT_1 receptors in cortical membranes was measured by a modification of the method described by Pedigo *et al.* (1981). The final pellet had been resuspended in 50 mM Tris-HCI buffer (pH 7.4), containing 4 mM $CaCI_2$. Incubations were in the same buffer containing 10 μM pargyline, 100 μl membrane suspension and ^{3}[H]5 hydroxytryptamine from 0.5–16 nM. The final volume was 1 ml. Each assay (10 min, 37°C) was performed in triplicate. Nonspecific binding was defined in the presence of 10 μM 5 hydroxytryptamine and was *ca.* 30% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]Ketanserin Binding

[^{3}H]Ketanserin binding to 5 HT_2 receptors in cortical membranes was measured by a modification of the method described by Leysen *et al.* (1986). The final pellet had been resuspended in 50 mM Tris-HCl buffer (pH 7.4). Incubations were in the same buffer, containing 100 μl membrane suspension and ^{3}[H]ketanserin from 0.25 8 nM. The final volume was 1 ml. Each assay (15 min, 37°C) was performed in triplicate. Nonspecific binding was defined in the presence of 1 μM methysergide and was *ca.* 20% of the total binding. Filtration, washing and scintillation spectroscopy were as described for [^{3}H]CHA.

[^{3}H]MK-801 Binding

[^{3}H]MK-801 binding to sites on NMDA receptors in cortical membranes was measured by a modification of the method described by Wong *et al.* (1986). The final pellet had been resuspended in 5 mM Tris-HCl buffer (pH 7.4). Incubations were in the same buffer, containing 100 μl membrane suspension and [^{3}H]MK 801 from 0.5–16 nM. The final volume was 1 ml. Each assay (45 min, 23°C) was performed in triplicate. Nonspecific binding was defined in the presence of 100 μM MK-801 and was *ca.* 5% of the total binding. Filtration, washing and scintillation spectroscopy were as described for ^{3}H]CHA.

[^{3}H]Nitrendipine Binding

[^{3}H]Nitrendipine binding to cortical membranes was measured by a modification of the method of Ehlert *et al.* (1982). The final pellet had been resuspended in 50mM Tris-HCl buffer (pH 7.4). Incubations were in the same buffer, containing l00 μl membrane suspension and [^{3}H]nitrendipine from 0.05–1 nM. The final volume was 1 ml. Each assay (90 min, 25°C) was performed the dark in triplicate. Nonspecific binding was defined in the presence of l μM and was *ca.* 10% of total binding. Filtration, washing and scintillation were as described for [^{3}H]CHA.

Data Analysis

The dissociation constant (K_D) and the maximal number of binding sites (B_{max}) were analyzed with the computer program LIGAND (Munson and Rodbard, 1980) for each experiment and for pooled data from at least three experiments and are reported as means ± SEM. The statistical evaluations were with the Student's t-test.

RESULTS

Chronic ingestion of caffeine for a period of 4 days by male NIH Swiss strain mice results in significant changes in densities of a variety of cortical or cerebellar receptors and in density

of cortical nitrendipine binding sites, presumed to be associated with L-type voltage sensitive calcium channels (Table I). Significant increases in B_{max} values in cortical membranes occur for the following: A_1 adenosine receptors (20%), $5HT_1$ and $5HT_2$ serotonergic receptors (26–30%), nicotinic and muscarinic receptors (40–50%), benzodiazepine binding sites associated with $GABA_A$ receptors (65%), and nitrendipine binding sites (18%) (Fig. 1). Only in the case of [^{3}H]diazepam binding to benzodiazepine modulatory sites was a significant change in K_D observed, namely a slight decrease in affinity (Table 1, $P < 0.05$). Significant decreases in B_{max} values occur for cortical β_1-adrenergic receptors (25%) and for cerebellar β_2 adrenergic receptors (27%). The B_{max} values for striatal A_{2A} adenosine receptors and D_1 and D_2 dopaminergic receptors and for cortical α_1 and α_2 adrenergic receptors and for [^{3}H]MK-801-channel sites associated with NMDA receptors were unchanged after chronic caffeine ingestion in male NIH Swiss strain mice.

DISCUSSION

The increase in density of cortical A_1 adenosine receptors after chronic blockade of such receptors by caffeine (Table 1) is not unexpected. Indeed, such have been previously reported by many laboratories primarily for rats (see INTRODUCTION). One report of no change in A_1-adenosine receptors in cerebral cortex after chronic caffeine has appeared (Holtzman *et al.,* 1991). In contrast to the up-regulation of A_1-adenosine receptors, there was no increase density of striatal A_{2A}-adenosine receptors after chronic blockade of such receptors by caffeine (Table 1). One study had reported no increase in striatal A_{2A}-adenosine receptors as measured by autoradiography (Lupica *et at.*, 1991), while another study reported a slight increase as measured by membrane binding assays(Hawkins *et al.,* 1988). In vitro in striatal slices A_1, but not A_{2A}-adenosine receptors undergo desensitization (Abbracchio *et al.,* 1992). In contrast, in vivo in rats an adenosine analog, NECA, down-regulated striatal A_{2A}-adenosine receptors, while having no effect on A_1 adenosine receptors (Portal *et al.,* 1998).

The increase in A_1 adenosine receptors in male NIH Swiss strain mice (Table 1) is accompanied by an increase in sensivity to the depressant effects of adenosine analogs on open-field locomotor activity (Nikodijević *et al., 1992).* Remarkably, this applies to both A_1 and A_2 selective analogs. There are synergistic effects on locomotor activity of A_1 and A_2 selective agonists (Nikodijević *et at.,* 1991) and thus endogenous adenosine through the A_1, receptors could enhance behavioral effects of A_2 selective adenosine analogs in control and chronic caffeine treated mice. It should be noted that male NIH Swiss strain mice do not become tolerant to the stimulatory effects of caffeine after chronic caffeine-ingestion (Nikodijević *et al.,* 1993).

Caffeine is not known to block extracellular receptors other than adenosine receptors at the dosage level (~100 mg/kg/day) employed in the present study. Thus, effects of chronic caffeine on levels of other central receptors are likely indirect. Certainly, blockade by chronic caffeine of the tonic input of endogeneous adenosine to presynaptic receptors that are inhibitory to neurotransmitter release (Williams, 1987) would be expected to alter turnover and hence postsynaptic activation of receptors for a variety of central neurotransmitters, including norepinephrine, dopamine, serotonin, acetylcholine, γ-aminobutyric acid and glutamate. Homeostatic regulation in such receptors or the second messenger systems subserved by such receptors might then be expected. The present results indicate that densities of some, but not all of such possible neurotransmitter receptors do significantly change as a result of 4 days of chronic caffeine ingestion (~100mg/kg/day) in male NIH Swiss strin mice.

The reduction on density of both β_1 and β_2 adrenergic receptors in cerebral cortex and cerebellum, respectively (Table 1), suggests a caffeine induced increase in turnover and action of norepinephrine at such receptors. Caffeine has been reported to increase norepinephrine turnover (Berkowitz *et al.,* 1970; Corrodi *et al.,* 1972; Hadfield and Milio, 1989). A down-regulation of cortical β_1 adrenergic receptors has been previously reported for rats after chronic caffeine (Goldberg *et al.,* 1982; Green and Stiles, 1986) or theophylline (Fredholm *et al.* 1984). The lack of change in α_1 and α_2 adrenergic receptors after chronic caffeine ingestion (Table 1) suggests either that these receptors exist mainly at sites or pathways whose noradrenergic input is unaffected by caffeine or that such receptors are not under homeostatic control in this strain of mice. In rat brain the densities of α_1, and α_2 adrenergic receptors were unaltered after chronic caffeine (Goldberg *et al.,* 1982). A single injection of pentoxifylline (3, 7-dimethyl-1-(5-oxohexyl)xanthine) caused a decrease in densities of both α_2 and β_1 adrenergic receptors in cortex of rats (Lowenstein *et al.,* 1982).

The lack of effect of chronic caffeine ingestion on dopamine receptors (Table 1) is remarkable in view of many reports, indicating a close interrelationship of adenosine and dopamine systems in striatum (see review by Ferré *et al.,* 1992). Thus, blockade of striatal adenosine receptors by caffeine enhances dopaminergic function (*cf.* Josselyn and Beninger, 1991 and references therein). Conversely, chronic central infusion of adenosine analogs in rats causes an attentuation in both A_{2A} adenosine and D_1 dopamine receptor elicited stimulation of striatal adenylate cyclase (Porter *et al.,* 1988). Other treatments, such as dopamine receptor blockade or denervation, do result in alterations in sensitivity to dopaminergic agonists (Yarbrough 1975; Siggins *et at.,* 1984; Chipkin *et al.,* 1984). Behaviorally, chronic ingestion of caffeine in the male NIH Swiss strain mice has no significant effect on stimulation of locomotor activity elicited by amphetamine, but does increase the maximal percent stimulation elicited by cocaine (Nikodijević *et al.,* 1993), agents thought to act by increasing dopaminergic function.

The densities of cortical $5HT_1$ and $5HT_2$-serotonin receptors are increased after chronic caffeine ingestion of male NIH Swiss strain mice (Table 1), suggesting a reduction in serotonergic input and a resultant up regulation of receptors. However, caffeine has been reported in some studies to *increase* rather than decrease serotonin turnover in rats (Berkowitz and Spector, 1971; Corrodi *et al.,* 1972) and mice (Valzelli *et al.,* 1973). Thus, a down regulation of serotonin receptors might have been expected. Behaviorally, effects of serotonergic agents do not appear to have been investigated after chronic caffeine.

The densities of cholinergic receptors, both nicotinic and muscarinic, appear increased after chronic caffeine ingestion, suggesting a decrease in acetylcholine function and a resultant up regulation of receptors. However, chronic treatment of rats with nicotine increases the density of nicotinic receptors (Ksir *et al.*, 1985; Hillard and Pound, 1993). Thus, caffeine might be increasing nicotinic receptors by enhancing acetylcholine release, rather than by decreasing release. Acute caffeine does increase acetylcholine release from rat cerebral cortex (Phillis *et al.,* 1980). Behaviorally, it appears that muscarinic function is enhanced in NIH Swiss strain mice after chronic caffeine ingestion, since higher doses of a muscarinic antagonist, scopolamine, are required to stimulate openfield locomotor activity compared to control mice (Nikodijević *et al.,* 1993). Both nicotinic (nicotine) and muscarinic (oxotremorine) agonists reduce openfield locomotor activity in control NIH Swiss strain mice, while being ineffective (nicotine) or less effective (oxotremorine) after chronic caffeine (Nikodijević *et at.,* 1993). This too could be interpreted as due to increase in cholinergic function, so that any further depressant effects of nicotine or oxotremorine are blunted. The mice do exhibit a marked long term reduction in openfield locomotor activity after chronic caffeine (Nikodijević *et al.,* 1993), which might reflect enhanced function of the up-regulated cholinergic receptors. Further studies directed towards such hypotheses are

required. Chronic caffeine has been reported to reduce the excitatory effects of acetylcholine on rat cerebral cortical neurons (Lin and Phillis, 1990). Chronic nicotine results in tolerance to depressant effects of nicotine in spite of regulation of nicotinic receptors (Ksir *et at.,* 1985).

The density of $GABA_A$ receptors, as assessed by binding of the $GABA_A$ modulator diazepam, is increased markedly in NIH strain mice (Table 1). There also appears to be a small but significant decrease in the affinity of diazepam; the nature of this decrease requires further investigation. Such an increase suggests an up-regulation of $GABA_A$ channels as a result of a decrease in GABAergic function during chronic caffeine treatment. An up regulation of benzodiazepine sites after chronic caffeine has been previously reported for rats (Wu and Coffin, (1984; Wu and Phillis, 1986). Chronic caffeine at a low dose of 10 mg/kg/day has little effect on diazepam-elicited changes in glucose utilization in brains of rat (Nehlig *et al.,* 1987). Chronic theophylline does decrease the convulsant activity of various agents in rats (Szot *et al.,* 1987), but whether this is due to up-regulation of adenosine receptors or an up regulation of $GABA_A$ receptors or is unknown.

The density of the NMDA class of glutamate receptors, as assessed by binding of the NMDA-channel blocker MK-1801, appears unchanged after chronic caffeine (Table 1). This is perhaps surprising in view of reports of functional : interrelationships of NMDA and adenosine receptors (Bartrup and Stone, 1990; Nagaoka *et al.,* 1993).

The density of L-type calcium channels, as assessed by binding of the L-type calcium channel antagonist nitrendipine is significantly increased in cortical membrances in male NIH Swiss strain mice after chronic caffeine ingestion (Table 1) The mechanism is unknown. Nitrendipine binding is also reported to be increased after chronic ethanol (Dolin *et al.,* 1987). Behavioral effects of calcium channel agents after chronic caffeine do not appear to have been assessed.

In summary, chronic ingestion of caffeine in male NIH Swiss strain mice results in increases in densities of A_1, but not A_{2A} adenosine receptors commensurate at least for the A_1 receptors with direct chronic blockade of such receptors by caffeine. Other G protein linked receptors also show changes in densities probably due to indirect effects of the blockade of adenosine receptors by caffeine on the tonic input of neurotransmitters to such G protein linked receptors. The β_1 and β_2 adrenergic receptors are down-regulated, while $5HT_1$ and $5HT_2$ serotonin receptors and muscarinic receptors are up-regulated. α_1 and α_2 Adrenergic receptors and D_1 and D_2 dopaminergic receptors are unaffected. Receptors that are part of a receptor-ion channel complex are also affected by chronic caffeine ingestion. The densities of the nicotinic receptor-channel and the $GABA_A$ receptor channel are increased, while the NMDA receptor challnel appears unchanged. The density of L-type calcium channels, as assessed by nitrendipine binding is increased by chronic caffeine ingestion. In rats, the density of forskolin-binding sites, presumably associated with adenylate cyclase, is increased after chronic caffeine (Daval *et al.,* 1989).

It appears likely that most alterations in receptors, ion channels and effector systems after chronic caffeine will prove related to blockade of tonic adenosine input by caffeine, rather than to blockade of phosphodiesterases or release of calcium from intracellular sites, since the latter two effects become significant only at much higher concentrations that the expected 10 μM brain concentrations of caffeine attained during chronic ingestion of caffeine (100 mg/kg/day) (see Ahlijanian and Takemori, 1986). It should be noted that phosphodiesterase inhibitors, including xanthines much more potent than caffeine do cause down-regulation of β-adrenergic receptors (Lowenstein *et al.,* 1982; Schultz and Schmidt, 1986). Further studies on behavioral responses of caffeine treated NIH Swiss strain mice (*cf.*

Nikodijević *et al.,* 1993) will be required to correlate changes in receptors and ion channels with caffeine induced tolerance, alteration in drug responsiveness, and withdrawal

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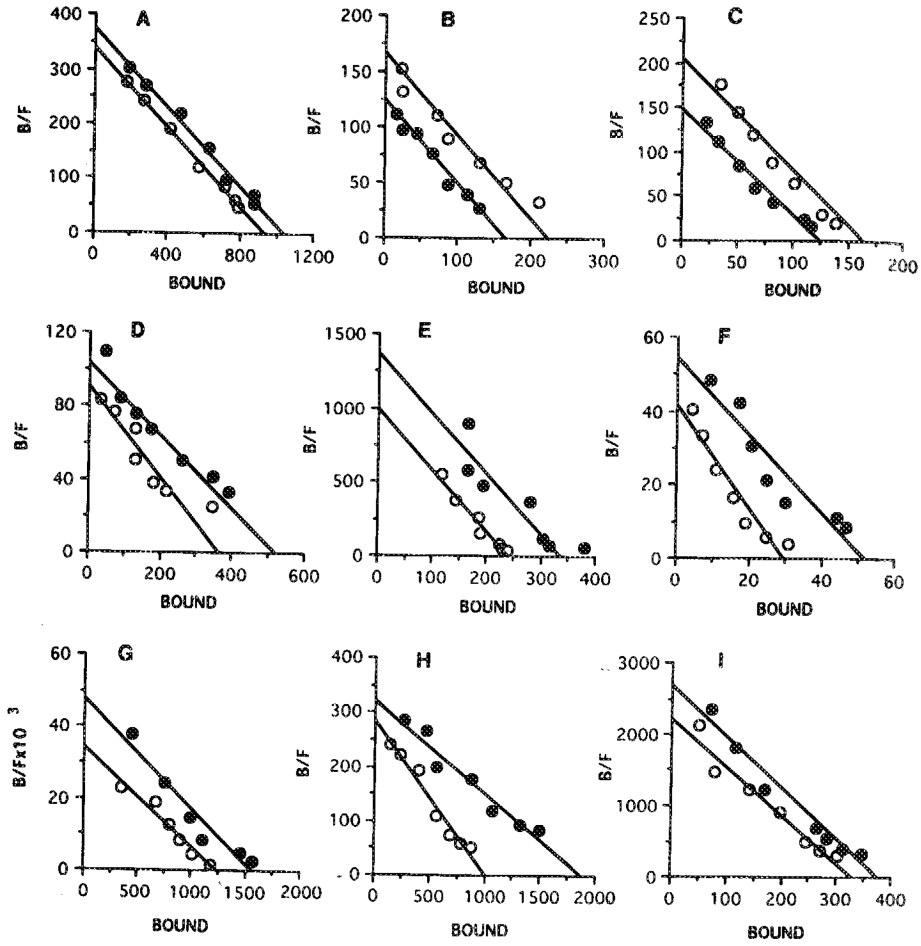

Fig. 1.

Scatchard Analyses for Alterations in Binding of Radioligands after Chronic Caffeine in NIH Swiss Strain Mice. Control, open circles; chronic caffeine, closed circles. A. Cortical A_1-adenosine receptor with [^{3}H]CHA. B. Cortical B_1,-adrenergic receptor with [^{3}H]DHA. C. Cerebellar β_2-adrenergic receptor with [^{3}H]DHA. D. Cortical 5-HT_1-serotonin receptor with [^{3}H]5-hydroxytryptamine. E. Cortical 5-HT_2-serotonin receptor with [^{3}H]5-ketanserin. F. Cortical nicotinic receptor with [^{3}H]nicotine. G. Cortical muscarinic receptor with [^{3}H]QNB. H. $GABA_A$ receptor with [^{3}H]diazepam. I. Calcium channel with [^{3}H]nitrendipine. Scatchard Analyses for the following were unchanged and are not shown: Striatal A_{2A}-adenosine receptor with [^{3}H]CGS 21680; Cortical α_1-adrenergic receptor with [^{3}H]prazosin; Cortical α_2-adrenergic receptor with [^{3}H]clonidine; Striatal D_1-dopaminergic receptor with [^{3}H]SCH 23390; Striatal D_2-dopaminergic receptor with [^{3}H]spiperone; Cortical NMDA receptor with [^{3}H]MK-801.

Table I

Effect of Chronic Caffeine Ingestion on Receptors and Ion Channels in Brain Membranes from Male NIH Swiss Strain Mice.

	K_d (nM)		B_{max}(fmol/mg protein)	
	Control	**Chronic Caffeine**	**Control**	**Chronic Caffeine**
A_1-Adenosine	1·95 ± 0.33	2.00 ± 0.45	911 ± 23	1089 ± 39**
A_{2A}-Adenosine	10.26 ± 0.66	9.98 ± 0.19	872 ± 57	884 ± 44
α_1-Adrenergic	0.049 ± 0.003	0.053 ± 0.012	175 ± 7	189 ± 12
α_2-Adrenergic	1.59 ± 0.20	1.37 ± 0.20	200 ± 3	193 ± 2
β_1-Adrenergic	1.15 ± 0.08	1.07 ± 0.14	224 ± 9	167 ± 5*
β_2- Adrenergic	1.57 ± 0.08	1.56 ± 0.10	158 ± 12	115 ± 11*
D_1-Dopamine	0.53 ±0.11	0.48 ± 0.06	3097 ± 81	3165 ± 66
D_2-Dopamine	0.108 ± 0.021	0.084 ± 0.018	729 ± 21	725 ± 55
5-HT_1	2.93 ± 0.37	3.35 ± 0.58	361 ± 14	474 ± 48**
5-HT_2	0.33 ± 0.09	0.35 ± 0.06	275 ± 11	347 ± 11*
Nicotinic	0.77 ± 0.05	0.95 ± 0.05	34 ± 2	50 ± 3*
Muscarinic	0.17 ± 0.02	0.17 ± 0.03	1153 ± 56	1509 ± 47**
NMDA (MK-801)	2.76 ± 0.29	2.66 ± 0.17	2653 ± 97	2588 ± 46
$GABA_A$ (diazepam)	2.82 ± 0.26	4.69 ± 0.50**	1061 ± 69	1741 ± 100*
Ca^{2+} Channel (nitrendipine)	0.16 ± 0.03	0.17 ± 0.02	314 ± 6	369 ± 12**

Kinetic analysis of binding of radioligands to cortical, cerebellar or striatal membranes from control mice and chronic caffeine-ingesting mice was carried out as described in METHODS. Values for K_D and B_{max} are means ± S.E.M. (n = 3 or greater).

* $P < 0.01$

** $P < 0.05$.