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ORIGINAL INVESTIGATION

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# $Ca^{2+}$ dependency of serotonin and dopamine release from CNS slices of chronically isolated rats

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**Abstract** Much evidence points to a significant involvement of the classical neurotransmitters 5-HT and DA in affective disorders with possible changes in different structures of the CNS and also at different levels of the signal transduction chain, i.e., receptor, synthesis, uptake or release. We have used chronic isolated housing as an animal model of depression. These isolated rats enabled the study of KCl-induced release of 5-HT and DA from nucleus accumbens, prefrontal cortex and hippocampal slices. The following questions were addressed: first, if there is a change in the depolarization dependent release of DA and 5-HT from these CNS structures, and second, if the release is through the classical exocytotic mechanism. A significant increase in KCl stimulated release of 5-HT was observed in chronically isolated animals when compared to controls. 5-HT release was completely abolished from controls or isolated animals, when slices were incubated with Krebs containing zero $Ca^{2+}$/10 mM $Mg^{2+}$, the inorganic $Ca^{2+}$ channel blockers, $Cd^{2+}$ or $Ni^{2+}$ and the calmodulin inhibitor, trifluoperazine. The organic $Ca^{2+}$ channel blockers nifedipine and D-600 were less effective in inhibiting the stimulated 5-HT release. KCl stimulated DA release was only significantly increased from hippocampus slices, of isolated, but not control animals. This release was also highly $Ca^{2+}$-dependent. The basal release of DA and 5-HT was similar in control and isolated animals and was not affected by the $Ca^{2+}$ channel antagonists. The results suggest that extracellular $Ca^{2+}$-dependent release of 5-HT and, to a lesser degree, of DA, is increased in this chronic animal model of depression in several CNS structures.

**Key words** Chronic isolation · Release · Exocytosis · 5-HT · DA · $Ca^{2+}$ · Hippocampus · Frontal cortex · Nucleus accumbens

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## Introduction

Serotonin (5-HT) and dopamine (DA) have been recognized as playing an important role in mood disorders and specifically in the etiology of depression (Jimerson 1987; Van der Kar 1989; Maes and Meltzer 1995; Willner 1995). Animal studies are helping to clarify the physiopathology of these affective disorders, and therefore several protocols have been established as possible models of depression (Willner 1984, 1990), one of them being chronically isolated housing.

Early isolation-rearing of animals has been known to induce several behavioral and neurochemical changes known as isolation syndrome, depending on species, sex, age and several environmental stimuli (Morgan et al. 1975; Valzelli 1977; Juraska et al. 1983; Moriman and Parker 1985). Thus isolated housing in rats enhances locomotor activity (Garzon and Del Rio 1981; Fulford and Marsden 1996) and stereotyped behavioral responses to dopaminergic drugs such as apomorphine and *d*-amphetamine (Sahakian et al. 1975) and increases the response to neuroleptic drugs (Sahakian and Robbins 1977). Isolation-rearing also increases the DA turnover in frontal cortexes of these animals (Blanc et al. 1980; Fulford and Marsden 1996) and the amphetamine-induced DA release and binding of $D_2$ receptors in the nucleus accumbens (Jones et al. 1990). Similarly, it changes the 5-HT turnover in isolated animals (Moriman and Parker 1985; Bickerdike et al. 1993) and causes prolonged in vivo release of 5-HT as well as of DA induced by KCl or fenfluramine, from the frontal cortex of isolated animals (Crespi et al. 1992). In all these studies, the animal isolation-rearing period, with partial sensory contact, ranged from 30 days to 1 year. It has been shown that early isolation-rearing, starting 21 days after birth, and lasting more than 9 months, induces hyperactivity in rats, when exposed to a novel environment, which can be reversed with antidepressant drugs (Garzon and del Rio 1981). This isolation protocol has been postulated to represent a satisfactory animal model of depression (Garzon and del Rio 1981; Willner 1990). Since isolated housing induces changes in the extracellular

concentration of DA and 5-HT, we were interested in studying the calcium dependency of the release of these two neurotransmitters in the chronic isolation model of depression to establish whether the exocytotic release mechanism of 5-HT and DA is modified in this model of depression. We have used structures of the limbic system, including prefrontal cortex (PFC), nucleus accumbens (N.Acc) and hippocampus (Hip), which are innervated by 5-HT (Azmitia and Segal 1978; Pazos et al. 1985) and DA terminals (Le Moal 1991).

## Materials and methods

### Animals

Male Sprague-Dawley (IVIC) rats were housed in individual cages and maintained on a 12-h light-dark cycle with free access to food and water.

Chronic isolation: As described by Garzon and del Rio (1981), newborn rats were isolated at 21 days after birth and kept in individual metallic cages (30×45 cm), but with auditory and olfactory contact with other animals for up to 12 months. Isolated animals were used for the experiments between 10 and 12 month of isolation. Control rats were kept in groups of five and used for the experiments at the same age as the isolated ones.

Test of locomotor activity: Animals were tested at 2 p.m. and placed in a box of white opaque plastic of 100×100 cm with 20×20 cm squares painted on the bottom of the box; the lid of the box was of clear plastic covered with mirror paper so that the animal could not see the observer. The animal was placed in the box and after 1 min of adaptation to the new environment, the locomotor activity was assessed by counting the squares crossed by the animal during 5 min. The box was cleaned after each animal was removed.

Microdissection: Animals were killed by decapitation between 10.00 and 13.00 hours. The brain was removed and placed in a Petri dish containing Krebs bicarbonate buffer (KB) of the following composition in mM: 125 NaCl; 5 KCl; 1.14 $MgSO_4$; 1.29 $KHPO_4$; 2 $CaCl_2$; 10 glucose; pH maintained at 7.4 by continuous oxygenation with $0_2/CO_2$ (95:5 w/w). Coronal slices of 400 µm of whole brain were obtained using a McIIlwain tissue chopper and the specific nuclei, i.e., N.Acc, PFC and Hip, were carefully free-hand microdissected according to the coordinates of the atlas of Paxinos and Watson (1982); the coordinates for N.Acc chosen were from bregma 2.2–1.2 mm and a square dissected out following the limits of the lateral ventricles and the caudate-putamen. For the PFC, the coordinates were from 3.2 to 2.2, and a triangle was dissected following the limits of the corpus callosum. For the HIP the coordinates were from 4.8 to 5.3, dissecting out the whole hippocampus from the slice separating the hippocampal fissure and the dentate gyrus. The complete procedure was performed at 4 ºC and immediately used for the study. For each incubation chamber, three slices of N.Acc. of approximately 3.20±0.30 mg wet weight, three slices of PFC (3.70±0.35 mg wet weight) and three slices of HIP (5.80±0.22 mg wet weight) were used. Each experimental condition was performed with a new set of slices.

Incubation and measurement of amines: Slices were placed in vials used as static perfusion chambers at 32 ºC and containing 500 µl oxygenated KB. Krebs was changed every 10 min for 30 min and tissue stimulated with 30 mM KCl for 10 min (Fig. 1). Only one concentration of KCl was used in this study, since in previous studies, this concentration showed to be the most suitable one to give a reliable release of neurotransmitter (Ayala and Jaffe 1993). The $Ca^{2+}$ antagonist was placed in the incubation medium 10 min before and during the first baseline (pre-stimulation period) and during the first KCl stimulation and the post-stimulation period and the outflow of catecholamines assessed. The outflow of the amines and their metabolites was measured as described by Ayala and Jaffe (1993), in pre-stimulation, stimulation and post-stimulation periods of 450 µl of the incubation media were acidified with 25 µl formic acid: water, 1:50 and centrifuged at 27000 g for 20 min. Supernatant was injected immediately into the HPLC system (Waters) using electrochemical detection and a 5 µm Supelcosil reverse face LC-18 column. The mobile phase was in mM: 1 EDTA; 0.65 octane sulfonic acid; 1 citric acid; 125 formic acid; 7.5% acetonitrile, pH adjusted with NaOH to 3.8 and filtered through nitrocellulose membrane 0.45 µm. Flow rate was 0.7 ml/min. The electrochemical system consisted of a glassy carbon electrode polarized at 0.72 V and an Ag/AgCl reference electrode. Concentrations were determined with the external standard method using a Waters integrator.

The basal release is expressed as the concentration of neurotransmitters during 10 min of the pre-stimulation period. The stimulated release is defined as the release during the stimulation and post-stimulation periods (10 min each) from which twice the basal release value is subtracted, and later divided by 2 to express stimulation during a 10-min stimulation. Stim. release=(stimulation period+post-stimulation period)–2×basal release/2. The results are presented as means±SEM and compared using Student's *t*-test or one-way ANOVA with different *n* values, after Sokal and Rohlf (1979).

Proteins were measured using the method of Bradford (1976).

### Drugs

Materials and drugs used were the following: NaCl, KCl, MgSO4, KHPO4, $CaCl_2$, glucose, $CdCl_2$, $NiCl_2$, D-600 (methoxyverapamil), Nifedipine (dissolved in dimethyl sulfoxide) from Sigma (St Louis, Mo., USA), trifluoperazine dihydrochloride from RBI (Natick, Mass., USA).

## Results

### Isolation of the animals

Animals were tested for locomotor activity after 11 months of isolation as described in the methods. Control rats, of the same age and weight as the isolated ones, showed an activity of 23.9±3.5 (*n*=9) squares crossed during 5 min, while the isolated rats showed an activity of 54.4±4.8 (*n*=10), squares crossed in 5 min ($P<0.005$).

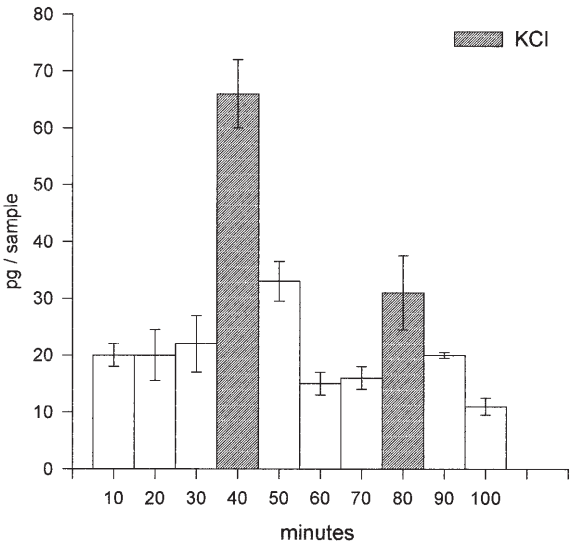


**Fig. 1** 5-HT release from nucleus accumbens slices from control animals. Slices were incubated and amines measured as described in methods. Slices were stimulated for 10 min with 30 mM KCl. Values are expressed in pg/500 µl sample, of ten experiments

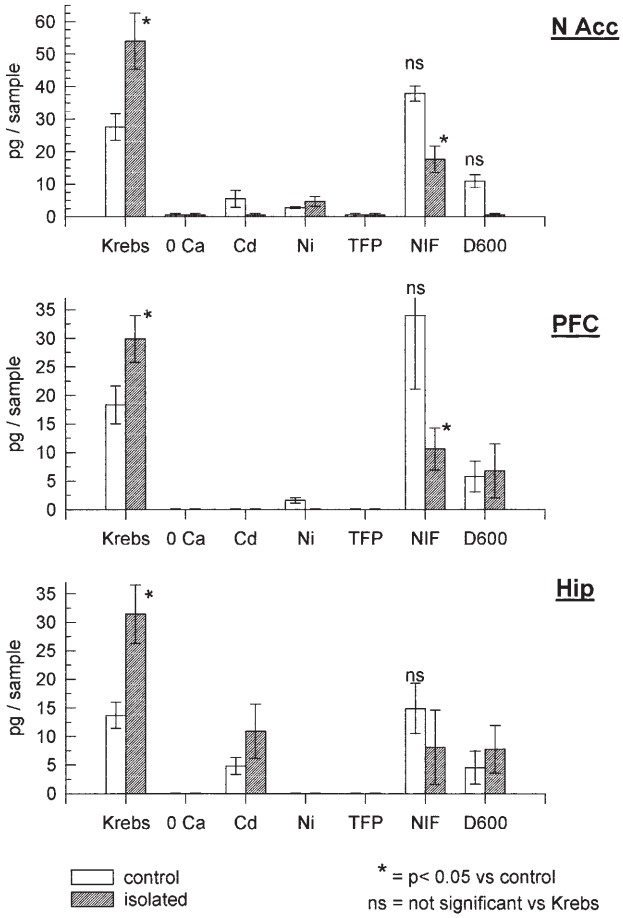


**Fig. 2** KCl stimulated release of 5-HT from control (*open bars*) and isolated rats (*filled bars*) from slices of nucleus accumbens (*N.Acc*), prefrontal cortex (*PFC*), and hippocampus (*Hip*). Basal release has been subtracted from stimulated release as described in methods. Slices were incubated in: Krebs: normal KB; zero Ca: zero $Ca^{2+}$/10 mM $Mg^{2+}$; $Cd^{2+}$:150 µM $Cd^{2+}$; $Ni^{2+}$: 100 µM $Ni^{2+}$; TFP: 100 µM trifluoperazine; NIF: 20 µM nifedipine; D600: 100 µM D-600. Values are expressed in pg/sample during 10 min of incubation. Each value is the mean±SEM of at least six experiments and are compared using Student's *t*-test and one-way ANOVA. Values from the different $Ca^{2+}$ modulating agents are all statistical significantly different compared to the values of the Krebs condition in a range of $P<0.05$ to $P<0.001$, if not indicated as not significant (ns)

## 5-HT release

After incubation of the slices during 30 min with three changes of KB, at 10-min intervals, a baseline release of 5-HT was reached (Fig. 1). The basal release from the N.Acc, PFC and Hip was similar in the three structures, about 5–20 pg/sample. No significant change in basal release was seen between isolated and control animals, and only a tendency in the inhibitory effect of the different $Ca^{2+}$ channel blocking conditions, showing no statistically significant $Ca^{2+}$ dependency.

Exposure of control slices to 30 mM KCl, for 10 min, caused a reproducible release of 5-HT from the three selected nuclei (Fig. 2). Isolated animals showed a significantly increased KCl stimulated release of 5-HT when compared to control rats, from the three structures studied. The release in all three structures from control, and from isolated animals, was highly $Ca^{2+}$ dependent, being 100% inhibited in zero $Ca^{2+}$/10 mM Mg. In the presence of 150 µM $Cd^{2+}$ or 100 µM $Ni^{2+}$, both inorganic inhibitors of the voltage sensitive $Ca^{2+}$ channels (Fox et al. 1987), a marked inhibition of the release was also observed, occasionally reaching 100% (Fig. 2). The organic $Ca^{2+}$ channel blockers, nifedipine (20 µM) and D-600 (100 µM), (Miller 1987) were less effective in inhibiting the KCl-stimulated release of 5-HT; nifedipine diminished the release of 5-HT from the three limbic structures in isolated but not in control animals (Fig. 2). D-600 diminished 5-HT release in control and in isolated animals. Interestingly, trifluoperazine (100 µM), considered being an inhibitor of calmodulin (Kenigsberg et al. 1982), and used as a potent antipsychotic agent, totally inhibited 5-HT release from the three selected structures (Fig. 2).

## DA release

The basal release of DA from the N.Acc, PFC and Hip ranged from 10 to 50 pg/sample, being highest in N.Acc. No significant change in basal release was seen between control and isolated animals and only a tendency in the inhibitory effect of the different $Ca^{2+}$ channel blocking conditions, showing no statistically significant $Ca^{2+}$ dependency.

Stimulation of the slices with 30 mM KCl induced a reproducible release of DA in the three structures in control and in isolated animals. Stimulated release from N.Acc was much higher (20- to 30-fold) than that from PFC and Hip (Fig. 3). Only in Hip was the stimulated DA release from isolated animals significantly higher, 192% versus controls. DA release from all three structures from control and from isolated animals was strongly $Ca^{2+}$ dependent, with 100% inhibition in zero $Ca^{2+}$/10 mM $Mg^{2+}$. In the presence of $Cd^{2+}$, $Ni^{2+}$, and TFP, the inhibition could also reach 100% (Fig. 3). Nifedipine and D-600 did not have a clear effect. Nifedipine appears to diminish the KCl-stimulated release of DA from Hip in isolated animals, while D-600 inhibited significantly the stimulated DA release from N.Acc of control animals (Fig. 3).

## Tissue content

Tissue content of 5-HT was similar in the three structures studied, around 2000 pg/mg protein in N.Acc, 1000 pg/mg protein in Hip and 3000 pg/mg protein in PFC, with no significant difference between control and isolated animals.

The tissue content of DA was 10 times higher in N.Acc (10000 pg/mg protein) than in PFC (1000 pg/mg protein) or Hip (400 pg/mg protein). However, no difference was seen between control and isolated animals. As

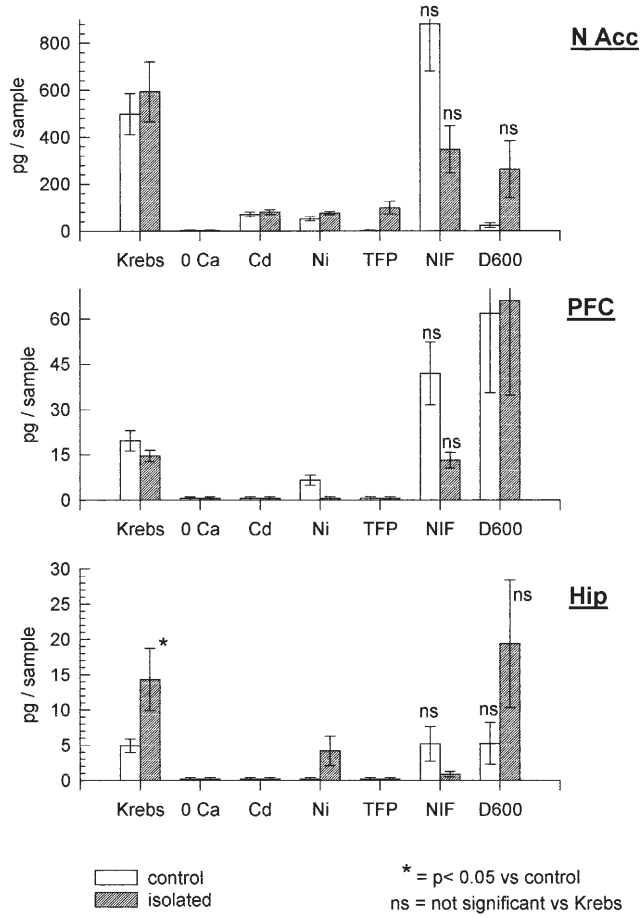


**Fig. 3** KCl stimulated release of DA from control (*open bars*) and isolated (*filled bars*) rats from slices of n. accumbens (*N.Acc*), prefrontal cortex (*PFC*), and hippocampus (*Hip*). Slices were incubated in different KB solution. Krebs: normal KB; zero Ca: zero $Ca^{2+}$/10 mM $Mg^{2+}$; $Cd^{2+}$:150 µM $Cd^{2+}$; $Ni^{2+}$: 100 µM $Ni^{2+}$; TFP: 100 µM trifluoperazine; NIF: 20 µM nifedipine; D600: 100 µM D-600. Values are expressed in pg/sample during 10 min of incubation. Each value is the mean±SEM of at least six experiments and all values are compared using Student's *t*-test and one-way ANOVA. Values from the different $Ca^{2+}$ modulating agents are all statistical significantly different to the values from the Krebs condition in a range of $P<0.05$ to $P<0.001$, if not indicated as not significant (ns).Only the Krebs condition in Hip showed a statistical difference between control versus isolated animals

a result, the change in release of 5-HT and DA from isolated animals could not be correlated to the tissue content of these amines.

## Discussion

Social isolation is considered one major cause contributing to the onset of depression. Long-term social isolation, with partial sensory contact, has been used as an animal model of depression (Garzon and del Rio 1981) and to assess the effectiveness of several antidepressants (Willner 1984).

Amines, specifically 5-HT and DA, have been associated with depression (Maes and Meltzer 1995; Willner 1995). Thus, it is believed that a deficit in serotonergic activity and diminished dopaminergic activity in certain brain areas may be associated with depression. However, little is known about the detailed neurochemical mechanisms underlying this condition.

In the present work, animals were isolated at the age of 21 days, with partial sensory contact, and after 11 months they showed a significantly different locomotor activity than controls, according to the results of Garzon and del Rio (1981).

This behavioral change was accompanied by a significant increase in the KCl stimulated, $Ca^{2+}$-dependent release of 5-HT in the isolated animals from the prefrontal cortex, nucleus accumbens and hippocampus. This finding is in apparent contrast with the widely held view that hypoactivity of the serotonergic system occurs in human depression (Maes and Meltzer 1995). Nevertheless, using different animal models of acute stress, anxiety or depression, a similarly increased release of 5-HT, and DA has been described. In fact, rats isolated for 1 month, starting 21 days after birth, showed an increased KCl stimulated release of DA and 5-HT from the frontal cortex measured by microdialysis technique (Crespi et al. 1992). Also, in the frontal cortex, increased 5-HT release could be observed in different animal models, including the forced swim stress (Jordan et al 1994), psychological stress in rats (Kawahara et al. 1993), conditioned fear (Yoshioka et al. 1995) and acute stress associated with learned helplessness (Petty et al. 1994).

Increased DA levels in nucleus accumbens have been observed following social isolation for more than 1 month (Jones et al. 1988) and restraint but not in animals restrained for 5 days (Imperato et al. 1992). Acute restraint (Shimizu et al. 1992) and learned helplessness (Edwards et al. 1992) increased KCl-stimulated 5-HT release from hippocampus, in vivo. In acutely restrained rats, desensitization of the $5HT_{1B}$ autoreceptors occurred, with no change in 5-HT release from hippocampus (Bolaños-Jimenez et al. 1995).Our results agree with most studies, pointing to a facilitation of the stimulated 5-HT release and, to a smaller extent, of DA release from limbic structures. The fact that the present findings of DA release from PFC did not correlate as well with some previous observations, for example, increased DA release from frontal cortex using in vivo microdialysis (Crespi et al. 1992), may be due to the different techniques used and that the changes observed are relatively small. Many of the previous studies have been done using in vivo microdialysis to assess the outflow of the amines; however, this study was performed in slice preparation, since under this condition, a more controlled environment can be obtained, and a better interaction of the different drugs at a cellular level; in this way, analyzing the actual modulation of the release at a specific site is easier. On the other hand, the slice preparation is less physiological than the intact animal preparation.

The facilitation of the release of 5-HT and DA could occur through different release mechanisms, as has been described for vesicle-mediated secretion (Bauerfeind

and Huttner 1993). The first of these could involve the constitutive secretory pathway common to all eukaryotic cells, responsible for the normal protein metabolism of the cell, which is only slightly $Ca^{2+}$ dependent (Girod et al. 1976). Secondly, 5-HT and DA release could take place through the regulated secretory granule pathway that is responsible for $Ca^{2+}$ dependent secretion of proteins and the dense core amine granules. Thirdly, the mechanism could involve the $Ca^{2+}$-dependent exocytosis of synaptic vesicles of neurons that mediate the release of neurotransmitters at the synapse (Jahn and Suedhof 1994). Lastly, release could be through the reversal of $Ca^{2+}$-independent neurotransmitter transporters (Attwell 1993).

To test whether this release occurs through the synaptic, extracellular $Ca^{2+}$-dependent mechanisms, we have used zero $Ca^{2+}$ media and $Ca^{2+}$ channel blocking agents known to inhibit most of the $Ca^{2+}$ channels described in the literature (Fox et al. 1987; Miller 1987; Reuter 1996). The results show that in control and isolated-reared animals release of 5-HT and DA is strongly $Ca^{2+}$-dependent, suggesting that it occurs through an exocytotic mechanism possibly involving different types of $Ca^{2+}$ channels (Nowycky et al. 1985; McCleskey 1994; Reuter 1996).

Trifluoperazine, a classical antipsychotic drug (Klein and Davis 1969), is a good inhibitor of calmodulin (Kenigsberg et al. 1982), a ubiquitous $Ca^{2+}$-binding protein (Brostom and Wolff 1981). Calmodulin is thought to regulate exocytosis through activation of the $Ca^{2+}$/calmodulin kinase II and interacting with proteins involved in exocytosis as synaptotagmin (Popoli 1993; Kibble and Burgoyne 1996). TFP was as efficient as the divalent cations, $Mg^{2+}$, $Cd^{2+}$ and $Ni^{2+}$ in blocking completely the release of 5-HT and DA from control and isolated animals. This reinforces our conclusions that in both control and isolated animals, DA and 5-HT release occurs via an extracellular release mechanism.

One point worth discussing is the apparently minor effectiveness of organic $Ca^{2+}$ antagonists (D-600 and nifedipine) as compared with divalent cations. Dihydropyridines (McCleskey 1994) have been used in psychopharmacology, with no effect on normal animals or humans, but with antidepressive or anxiolytic characteristics in some animal models (Pucilowski 1992). In agreement with this, we have found that nifedipine and D-600 were more effective in reducing the stimulated release of 5-HT from isolated than from control animals. This might suggest, that L type $Ca^{2+}$ channels, selectively blocked by these organic $Ca^{2+}$-blockers, are not so clearly associated with the release machinery (McCleskey 1994; Reuter 1996); they may be involved in some regulatory step, before exocytosis, evident only in isolated animals.

In conclusion, the involvement of voltage-dependent $Ca^{2+}$ channels and Calmodulin dependent increased release of 5-HT and DA from prefrontal cortex, hippocampus and nucleus accumbens could be shown in an animal model of depression, as chronic social isolation.

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