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Research Report

Cannabinoid system in the budgerigar brain

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ARTICLE INFO

Article history:
Accepted 28 February 2006
Available online 13 April 2006

Keywords:
CB_1 receptor
WIN55,212-2
SR141716A
Receptor autoradiography
[^{35}S]GTPγS autoradiography
avian

ABSTRACT

Cannabinoid receptor density and cannabinoid receptor-mediated G protein stimulation were studied by autoradiographic techniques throughout the budgerigar (*Melopsittacus undulatus*) brain. The maximal CB_1 receptor density value (using [^{3}H]CP55,940 as radioligand) was found in the molecular layer of the cerebellum (Mol), and high binding values were observed in the nucleus taeniae amygdalae (TnA), nucleus preopticus medialis, and nucleus pretectalis. The highest net-stimulated [^{35}S]GTPγS binding values induced by the selective CB_1 receptor agonist WIN55,212-2 were observed in the nucleus paramedianus internus thalami, and high values of [^{35}S]GTPγS binding were observed in the TnA, Mol, arcopallium dorsale and arcopallium intermedium. The distribution data suggest that in the budgerigar, as previously indicated in mammals, cannabinoid receptors may be related to the control of several brain functions in the motor system, memory, visual system, and reproductive behavior. The discrepancies between the cannabinoid receptor densities and the cannabinoid receptor-mediated stimulation found in several budgerigar brain nuclei support the hypothesis, previously described for mammals, of the existence of different $G_{i/o}$ protein populations able to associate with the cannabinoid receptors, depending on the brain structure, and could reflect the relative importance that cannabinoid transmission could exerts in each cerebral area.

1. Introduction

In recent years, the study of the cannabinoid system in the brain has received much attention because of the widespread and complex effects on higher cognitive functions exerted by cannabis (*Cannabis sativa*). Two different cannabinoid receptor subtypes, CB_1 and CB_2, have been described (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993; Piomelli et al., 2000). These receptors have been found to be coupled to $G_{i/o}$ proteins (Howlett et al., 1986, 1988).

Although the CB_1 receptor is the predominant subtype in the central nervous system (CNS) in mammals, the presence of the CB_2 subtype has also recently demonstrated (Van Sickle et al., 2005). CB_1 receptor is highly expressed in cerebral cortex, hippocampus, basal ganglia, and cerebellum (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992a; Glass and Felder, 1997). Interestingly, this subtype has been also found in striatal astrocytes (Rodriguez et al., 2001). Despite the conserved presence of this receptor in the CNS, different patterns of CB_1 receptor distribution have been found between

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doi:10.1016/j.brainres.2006.02.119

humans and rodents. Thus, a significantly higher density of this receptor in the human amygdala and cingulate cortex as compared with those of rat and monkey has been described in the same brain areas (Herkenham et al., 1990). In addition to the reported presence in the brainstem, the distribution of the CB_2 receptor indicates that this subtype is primarily localized on cells related to the immune system, in particular, mature B cells and macrophages (Galiegue et al., 1995). Regarding studies on avian species, few authors have addressed cannabinoid receptors in birds, and no detailed distribution of these receptors has been provided (Soderstrom and Johnson, 2000). CB_1 receptors seem to be the only type of cannabinoid site in the CNS of birds (Soderstrom and Johnson, 2000), although a CB_2-like protein has been also described in the CNS in chick embryos but not in adult chickens (Fowler et al., 2001).

The type of neurons displaying the CB_1 receptor has been described to be efferent striatal GABAergic neurons and striatum–nigral and striatum–pallidal neurons, releasing substance P and enkephalins, respectively (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992b). Similarly, the existence of this subtype of receptor on hippocampal GABAergic interneurons (Tsou et al., 1998) and in cerebellar glutamatergic granular neurons has been reported (Levenes et al., 1998). Furthermore, the reported presynaptic localization of these receptors is consistent with the proposed role of endogenous cannabinoid compounds released by postsynaptic neurons as modulators of the release of excitatory and inhibitory neurotransmitters by presynaptic terminals (Hoffman and Lupica, 2000; Maejima et al., 2001; Diana et al., 2002). This proposed modulator role, together with the existence of very high densities of CB_1 receptors through the CNS, supports the relevance of the endocannabinoid system as a general mechanism of central regulation (Freund et al., 2003).

The budgerigar (*Melopsittacus undulatus*) belongs to the order Psittaciformes, which together with the Oscines and Trochiliformes are avian orders that contains vocal learning species (Ball and Hulse, 1998; Gahr, 2000; Jarvis et al., 2000; Janata, 2001; Roberts et al., 2001). Molecular and cladistic analyses provide evidences that vocal learning has evolved independently in these orders (Striedter, 1994). Species of the order Psittaciformes present a well-developed song control system which has recently been described (Durand et al., 1997) to show differences with that of the Oscines, as well as some differences in the functions of the song system, including the ability to imitate sounds (Hile et al., 2000; Plummer and Striedter, 2000).

The level of functionality of GPCRs can now be analyzed in deep by means of [^{35}S]GTPγS binding assays. In addition, a high density of membrane receptors does not always imply a high level of signal transduction. Because of that and taking into account the very limited information available on the presence and activity of cannabinoid receptors in the avian brain, a detailed study on the distribution of CB_1 receptor density and functionality (transductional properties) in the brain of budgerigar could provide important information about both the general role of the endocannabinoid system in birds nervous system and the specific involvement of these receptors in the regulation of song control systems.

Here, we present, for the first time, a complete and detailed distribution of the CB_1 receptor protein, together with the distribution of the degree of functionality mediated by this receptor, throughout the brain of this bird species.

2. Results

2.1. *Functional autoradiography*

Preliminary experiments were performed to determine the optimal concentrations of WIN55,212-2 able to stimulate [^{35}S]GTPγS binding mediated by cannabinoid receptors (unpublished results). In this sense, most reports have used 10 μM WIN55,212-2 for cannabinoid stimulation in functional autoradiographic assays using rat brain tissues (Sim et al., 1995; Berrendero et al., 1998; Breivogel et al., 1997). However, the need for higher concentrations (100 μM WIN55,212-2) for reaching maximal stimulation in the human brain has been reported (Rodriguez-Puertas et al., 2000). Our data using 10 μM and 100 μM WIN55,212-2 showed that both concentrations elicited maximal stimulation in budgerigar brain tissues. We used an agonist concentration of 10 μM for the distribution described here to maintain the standard protocols described for rat.

2.2. *Basal [^{35}S]GTPγS binding*

The highest basal [^{35}S]GTPγS binding values were found in the diencephalic nucleus preopticus medialis (POM). Very high levels of basal binding (>75% with respect to the structure of maximal basal binding value, POM) were found in some telencephalic structures such as nucleus striae terminalis lateralis (NSTL), and in the following diencephalic structures: nucleus dorsomedialis posterior thalami (DMP), nuclei habenularis medialis and lateralis (HM and HL). High levels of basal binding (50 to 75% with respect to POM) were found in nucleus paramedianus internus thalami (PMI) (diencephalon). The remaining structures showed moderate (25 to 50% with respect to POM) or low (0 to 25% with respect to POM) basal [^{35}S]GTPγS binding values. See Table 1 and Fig. 1.

2.3. *Total agonist-stimulated [^{35}S]GTPγS binding*

Total agonist-stimulated [^{35}S]GTPγS binding values obtained in the presence of the cannabinoid agonist WIN55,212-2 are shown in Table 1 and Fig. 1. The highest [^{35}S]GTPγS binding value in this condition was found in the diencephalic PMI nucleus. Very high [^{35}S]GTPγS binding values (>75% with respect to the structure of maximal density in the brain, the PMI) were found in the nucleus taeniae amygdalae (TnA) and NSTL (telencephalon) as well as in the POM, DMP, HM and HL (diencephalon). High binding levels (50 to 75% with respect to the PMI) were found in the hyperpallium apicale (HA), mesopallium (M), nucleus centralis nidopallii lateralis (NLC), nidopallium intermedium (NI), robust nucleus arcopallialis (RA), arcopallium dorsale (AD), arcopalium intemedium (AI), hippocampus (Hp) and area parahippocampalis (APH) (telencephalon), the nucleus superficialis parvocellularis (SPC) (diencephalon), the stratum griseum periventriculare (SGP) (mesencephalon), and

Table 1 – Receptor and cannabinoid agonist-stimulated binding values throughout the budgerigar brain

	[^{3}H]CP55,940 binding values	Basal [^{35}S]GTPγS binding values	Agonist-induced [^{35}S]GTPγS binding values
	Mean ± SEM	Mean ± SEM	Mean ± SEM
HA	83 ± 1	151 ± 2	410 ± 91
HD	81 ± 2	143 ± 20	353 ± 48
M	78 ± 2	174 ± 11	506 ± 44
N	122 ± 2	85 ± 48	319 ± 33
NLC	149 ± 6	111 ± 29	414 ± 24
NC	113 ± 5	114 ± 9	365 ± 20
NI	70 ± 5	116 ± 7	493 ± 5
mpStM	153 ± 7	143 ± 29	357 ± 65
lpStM	200 ± 6		
StMm	151 ± 8		
PVt	195 ± 8	45 ± 2	96 ± 13
E	75 ± 7	68 ± 7	189 ± 15
PB	126 ± 10		
StL	99 ± 2	139 ± 16	263 ± 42
RA	218 ± 5	124 ± 40	477 ± 32
AD	169 ± 13	137 ± 28	546 ± 65
AI	167 ± 20	159 ± 37	572 ± 83
TnA	267 ± 14	132 ± 10	636 ± 61
NSTL	204 ± 9	484 ± 45	733 ± 37
Hp	200 ± 7	172 ± 25	516 ± 16
APH	120 ± 13	210 ± 30	480 ± 40
L	138 ± 11	119 ± 58	260 ± 38
POM	256 ± 11	506 ± 52	697 ± 28
DMm	156 ± 6	40 ± 10	173 ± 10
Rt	240 ± 7	126 ± 10	278 ± 23
DIP	72 ± 5	164 ± 12	265 ± 17
DMP	128 ± 12	409 ± 30	650 ± 40
HM	144 ± 11	480 ± 49	780 ± 58
HL	117 ± 9	427 ± 25	724 ± 23
SPC	83 ± 2	223 ± 15	521 ± 20
PMI	161 ± 7	277 ± 31	804 ± 18
PT inner area	246 ± 5	41 ± 4	275 ± 30
PT outer area		185 ± 13	540 ± 55
SGFS	54 ± 2	173 ± 14	242 ± 14
SGC	108 ± 6	116 ± 2	183 ± 11
SAC	38 ± 4	36 ± 6	66 ± 5
SGP	218 ± 11	188 ± 5	404 ± 5
MLd	62 ± 3	108 ± 12	174 ± 21
Ico	202 ± 10	187 ± 14	376 ± 62
Imc	38 ± 2	70 ± 8	75 ± 9
Ipc	43 ± 4		
OMd	23 ± 1	29 ± 2	52 ± 8
Mol	376 ± 12	70 ± 3	485 ± 22
Gra	37 ± 2	52 ± 17	117 ± 15

Receptor autoradiography binding values using [^{3}H]CP55,940 are expressed as fmol radioligand/mg equivalent tissue. Basal and agonist-stimulated [^{35}S]GTPγS binding values are expressed as nCi/g equivalent tissue.

the molecular layer of the cerebellum (Mol). Moderate [^{35}S]GTPγS binding values (25 to 50% with respect to the PMI) were observed in the hyperpallium densocellulare (HD), nidopallium (N), lateral nidopallium (NL), striatum mediale, lateral part of the striatum mediale (lpStM), striatum laterale (StL) and field L (L) (telencephalon), nucleus rotundus (Rt), and nucleus dorsointermedius posterior thalami (DIP) (diencephalon) as well as nucleus pretectalis (PT), stratum griseum et fibrosum superficiale (SGFS), and nucleus intercollicularis (Ico) (mesencephalon). The remaining structures had low [^{35}S]GTPγS binding values (<25% with respect to the PMI).

2.4. *Specificity of cannabinoid-stimulated [^{35}S]GTPγS binding*

[^{35}S]GTPγS binding in the presence of the agonist WIN55,212-2 (10 μM) and the antagonist SR141716A (10 μM) displayed similar values to those found for basal [^{35}S]GTPγS binding.

2.5. *Receptor autoradiography*

The distribution of [^{3}H]CP55,940 binding values throughout the budgerigar brain is shown in Table 1, and microphotographs from representative encephalic levels are shown in Fig. 2. The highest binding level was found in the molecular layer of the cerebellum (Mol) and the remaining structures of the brain presented binding values 75% lower than that observed in Mol. The lateral part of the striatum mediale (lpStM), pallidum ventrale (PVt), RA, TnA, NSTL and Hp in the telencephalon, the POM and Rt in the diencephalon as well as the PT, SGP and Ico in the mesencephalon displayed high binding values (ranging between 50 and 75% with respect to Mol). Several structures of the telencephalon, including N, NLC, NL, medial part of the striatum mediale (mpStM), striatum mediale, pars magnocellularis (StMm), perientopallial belt (PB), AD, AI, APH, and L and some structures of the diencephalon such as magnocellular nucleus of the dorsomedial thalamus (DMm), DMP, HM, HL, and PMI, displayed moderate binding values (ranging between 30–50% with respect to Mol). The remaining structures studied displayed [^{3}H]CP55,940 binding values lower than 30% and were considered to display low or non-specific binding.

2.6. *Correlation between receptor and functional autoradiography*

Correlation analyses between CB_1 receptor densities (measured as [^{3}H]CP55,940 binding values) and CB_1 receptor-mediated stimulation of G proteins (net agonist-stimulated [^{35}S]GTPγS binding) showed a Pearson correlation factor of $r = 0.5382$ ($P < 0.001$) when all the brain structures studied were included. However, analyses of the structures belonging to each main brain region indicated that the mesencephalon presented a Pearson factor of $r = 0.9488$ ($P < 0.001$), the telencephalon correlation factor was $r = 0.3431$ ($P = 0.12$), and the diencephalon was $r = -0.0920$ ($P = 0.81$).

Net [^{35}S]GTPγS/[^{3}H]CP55,940 binding ratios differed among brain structures (Fig. 3). Many brain areas had ratio values close to 1, including all the structures with the highest values of receptor binding. However, some structures, such as HA, M, NI, SPC, or PMI, with moderate or low receptor binding values, presented ratios higher than 3. In

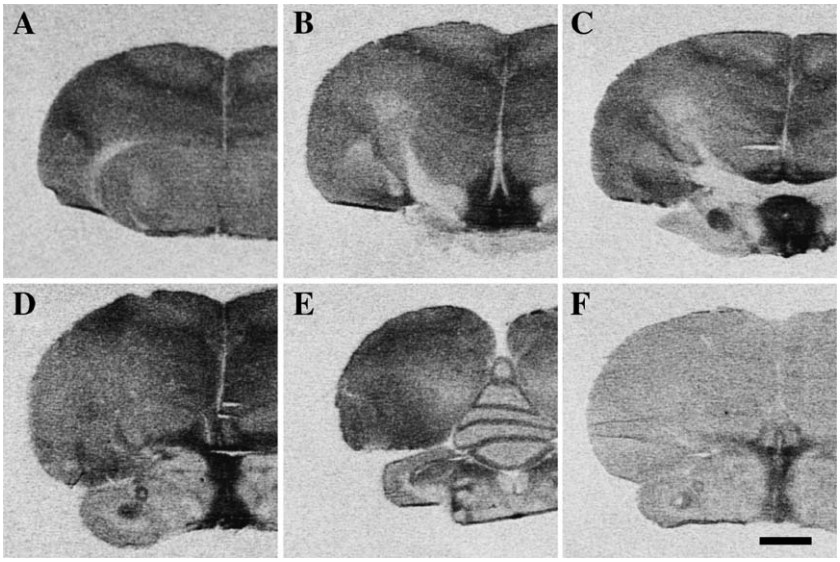

Fig. 1 – Autoradiographic images of WIN55,212-2-stimulated [^{35}S]GTPγS binding at different budgerigar brain levels (A–E, see corresponding drawings in Fig. 2). A representative image of basal binding corresponding to panel D level is shown in panel F. Scale bar: 2 mm.

contrast, structures such as VP, with high receptor binding values, displayed very low net stimulations (ratio = 0.25).

3. Discussion

Our functional autoradiographic assays using the selective CB_1 antagonist SR141116A completely inhibited the stimulation of G proteins induced by the non-specific cannabinoid agonist WIN55,212-2 at both concentrations used, and therefore, in the budgerigar brain, we were unable to detect $G_{i/o}$ protein stimulation mediated through CB_2 receptors using concentrations of up to 100 μM of this agonist. These results suggest that functional CB_2 receptors are probably not present in the adult budgerigar brain, as has been described for mammals (Howlett et al., 2004), and in accordance with data indicating the absence of CB_2 cannabinoid receptors in the adult chicken brain (Fowler et al., 2001).

The heterogeneous distribution of the [^{3}H]CP55,940 binding found throughout the budgerigar brain is roughly in agreement with that reported for mammals (Herkenham et al., 1990; Howlett et al., 2004). Also, a general agreement between our cannabinoid-stimulated [^{35}S]GTPγS binding data in budgerigar structures and their mammalian corresponding structures (Breivogel et al., 1997; Rodriguez-Puertas et al., 2000) can be observed. Thus, both the receptor density and receptor-mediated stimulation data suggest similar roles for cannabinoid receptors in birds and mammals. However, discrepancies appeared in some structures, suggesting evolutionary differences, as discussed below.

3.1. *Cerebellum*

In the cerebellum, very high amounts of CB_1 receptors have been reported in rodents using Western blot assays (Tsou et al., 1998) and "in vivo" autoradiography techniques (Gatley et al., 1998). Regarding our [^{3}H]CP55,940 binding studies, the most outstanding area in the budgerigar brain is the molecular layer of the cerebellum, which by far displays the highest density of cannabinoid receptors with respect to the remaining areas of the brain. These data contrast with those reported in mammals, where the cerebellum also showed high – but not maximal – cannabinoid receptor densities (Herkenham et al., 1991). With respect to agonist WIN55,212-2 G protein activation, the budgerigar cerebellum showed high cannabinoid stimulation values, in accordance with the high receptor density found in this structure. However, the agonist stimulation in the budgerigar cerebellum was similar to that of other structures with moderate cannabinoid receptor densities. In mammals, the correspondence between high [^{3}H]CP55,940 binding (Herkenham et al., 1990; 1991) and high cannabinoid stimulation values in this structure (Breivogel et al., 1997) has been described in the rat. Otherwise, the relatively low densities of cannabinoid receptors in cerebellum reported for humans (Herkenham et al., 1990; 1991) contrast with the high cannabinoid stimulation of G protein observed (Rodriguez-Puertas et al., 2000). There is thus a lack of proportionality between receptor density and G protein stimulation in the cerebellum in both birds and mammals. This suggests that differences in the efficiency of the biochemical transduction mechanism, depending on the structure and/or species, could exist.

3.2. *Basal ganglia*

Some mammalian basal ganglia such as the globus pallidus, caudate-putamen, substantia nigra pars reticulata, and nucleus entopeduncular have been reported to show high densities of cannabinoid receptors (Herkenham et al., 1991), which agrees with the high cannabinoid receptor binding observed in the budgerigar ventral pallidum. In contrast, other budgerigar basal ganglia nuclei, such as the lateral striatum, had moderate cannabinoid receptor densities. Similar

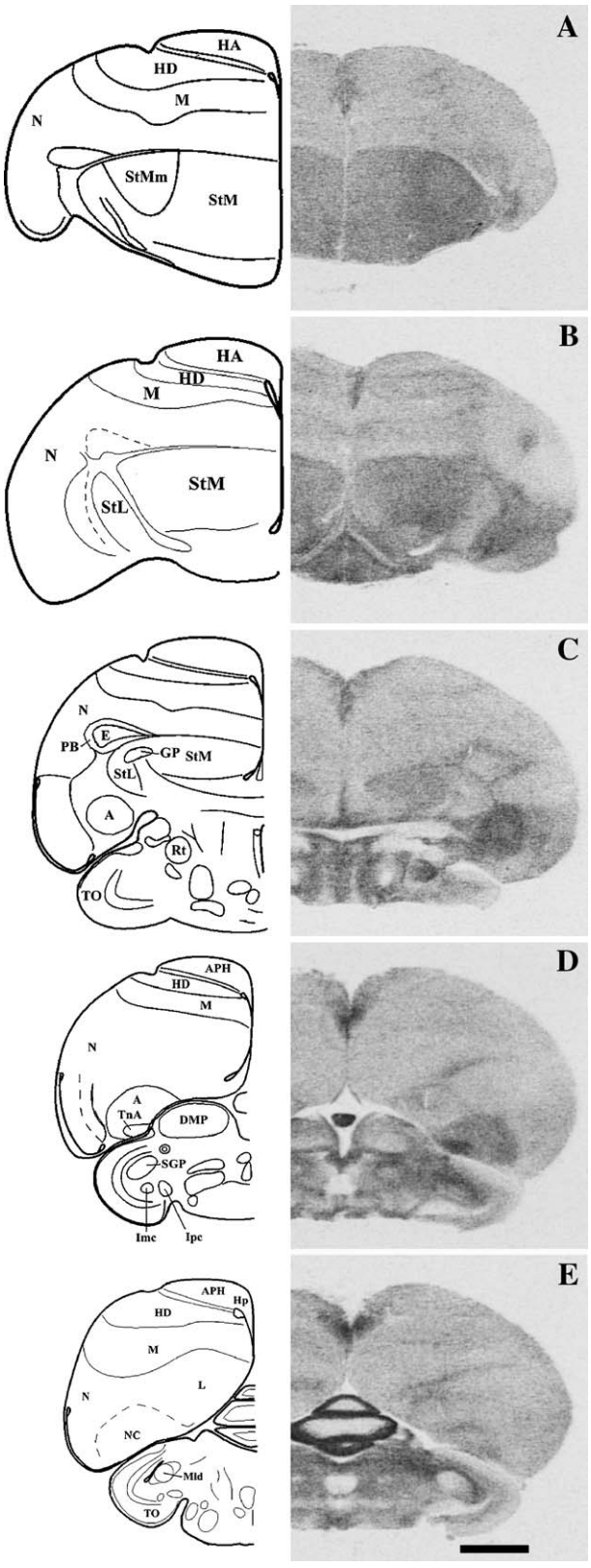

Fig. 2 – Autoradiographic images of receptor binding using [^{3}H]CP55,940 at different budgerigar brain levels. Drawings in the left side depict the main structures corresponding to the autoradiographs. Abbreviations are listed at the beginning. Scale bar: 2 mm.

cannabinoid-stimulated [^{35}S]GTPγS binding values for rat cerebellum and striatum (Breivogel et al., 1997) and higher values in the human basal striatum than in the cerebellum (Rodriguez-Puertas et al., 2000) have been described. On the other hand, our data indicate low net stimulation values in the budgerigar lateral striatum and ventral pallidum in contrast with the high levels of cannabinoid receptors in the ventral pallidum. Comparison of the data from mammals and the budgerigar suggests evolutionary differences in the role of cannabinoid receptors in the functions of the striatum. Cannabinoid receptors have been suggested to support an important role in the motor functions on the basis of their density in the molecular layer of the cerebellum and basal ganglia in mammals, as well as the motor depression induced by cannabinoid drugs (Herkenham et al., 1990). In this sense, our data also suggest an important role for the cannabinoid receptor in budgerigar motor control functions.

3.3. *Hippocampus*

The budgerigar hippocampus showed a correlation between [^{3}H]CP55,940 binding densities and cannabinoid-mediated G protein stimulation values similar to that observed in the cerebellum. Interestingly, in mammals, the correlation is different in the different species reported. Thus, rat cannabinoid receptor densities (Herkenham et al., 1990) and cannabinoid-stimulated [^{35}S]GTPγS values (Breivogel et al., 1997) have been described at a ratio comparable to that observed in our budgerigar study. In humans, however, the moderate cannabinoid receptor densities (Herkenham et al., 1990) show very high cannabinoid stimulation values (Rodriguez-Puertas et al., 2000), suggesting that the importance of cannabinoid transmission may differ, depending on the species, always assuming that G protein stimulation mirrors the efficiency of transmission in a more accurate way than receptor density. With this assumption, cannabinoid transmission in the hippocampus would be more important in humans than in the rat or budgerigar, while in the cerebellum, these differences would not be so striking. Thus, the cannabinoid system would appear to be important for motor learning in both mammalian and avian species. Thus, the cannabinoid system would appear to be important for motor learning in both mammalian and avian species. In contrast, the relevance of cannabinoid receptors in the hippocampus, a structure important for forming new declarative memories (Iversen, 2003), apparently differs considerably among avian and mammalian species.

3.4. *Other brain areas*

Some areas show discrepancies between mammals and birds. Very high values of [^{3}H]CP55,940 binding are found in the mammalian substantia nigra and globus pallidus (Herkenham et al., 1990, 1991), while only high values are observed in their putative avian homologues (Reiner et al., 2004). In contrast, the moderate [^{3}H]CP55,940 binding values observed in the mammalian amygdala complex (Herkenham et al., 1990, 1991) do not agree with the high values observed in the avian homologues AD and TnA (Reiner et al., 2004).

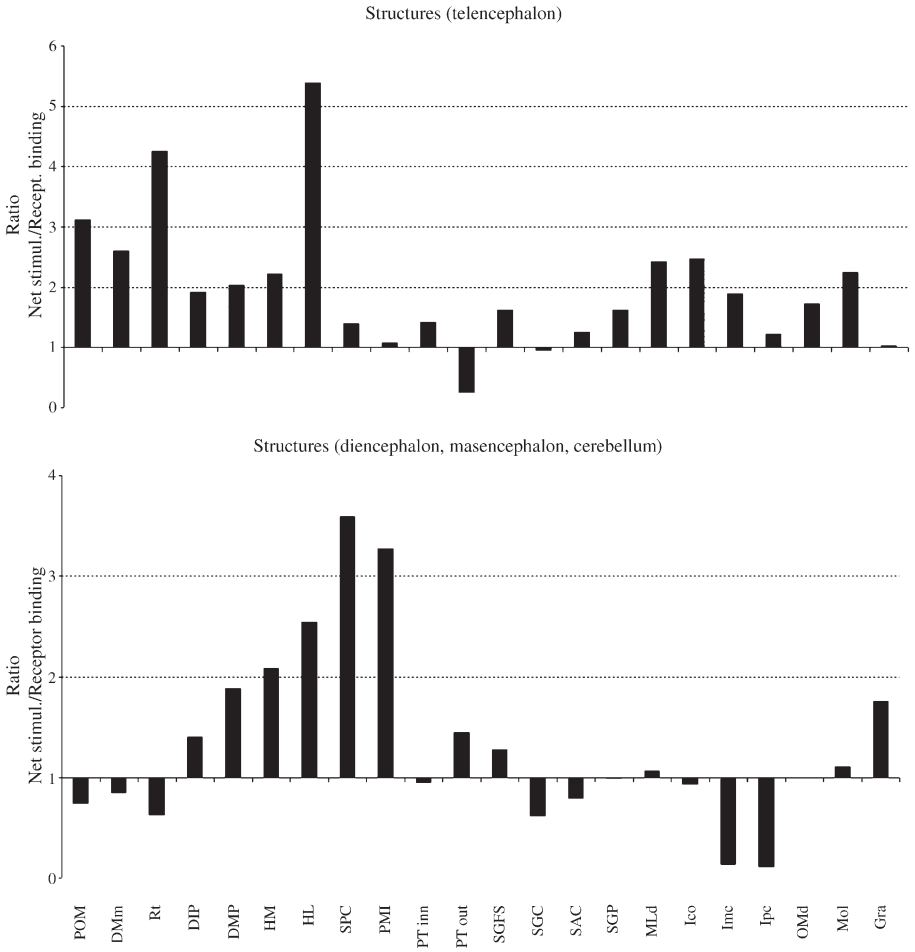

Fig. 3 – Ratio between net agonist-stimulation of [^{35}S]GTPγS mediated by CB receptors (data expressed as nCi/g eq. tissue) and CB receptor binding using [^{3}H]CP55,940 as radioligand (fmol/mg eq. tissue) of the different brain structures.

Interestingly, some nuclei related to the visual system, such as the nucleus rotundus and the nucleus pretectalis (Theiss et al., 2003) as well as some nuclei related with the song system, such as the StMm or the robust nucleus of the arcopallium (Durand et al., 1997; Jarvis and Mello, 2000), showed high cannabinoid receptor levels and some of them (i.e., PT, StMm, RA) also showed high net [^{35}S]GTPγS binding stimulation levels. As some evidences suggest the existence of gender dimorphism in the volumes of vocal nuclei (Brauth et al., 2005), the results here reported for those nuclei have to be analyzed taking into account that this study has been carried out in male subjects. Thus, our findings support the previously reported important role of cannabinoid receptors in the song system (Soderstrom and Johnson, 2003; Soderstrom and Tian, 2004) and suggest the involvement of these receptors in the visual system.

Cannabinoid drugs (tetrahydrocannabinol) have also been related to sexual behavior in mammals (Hernandez-Tristan and Arevalo, 1999). In quail, the POM and NSTL have been reported to be involved in the control of male sexual behavior (Balthazart et al., 1998). Our data in the budgerigar show that these nuclei have high levels of cannabinoid receptors and moderate G protein stimulation levels, thus supporting a possible role for cannabinoid receptors in male sexual behavior in this species as well.

3.5. *CB receptor-mediated G protein activation efficiency*

An interesting question emerging from this study is the difference observed in the net agonist-stimulation of [^{35}S]GTPγS mediated by CB receptors/CB receptor binding ratio in structures with moderate or low receptor binding values, which presented net stimulations much higher than expected (HA, M, NI, SPC or PMI). These areas seem to elicit very high stimulation from a relatively low presence of receptors. On the other hand, the VP with high receptor binding values showed very low net stimulation. This type of discrepancy has also been detected in mammals (Breivogel et al., 1997), in which it has been suggested that $G_{i/o}$ alpha subunits ($G_1\alpha_1$, $G_1\alpha_2$, $G_1\alpha_3$, $G_{o\alpha 1}$, $G_{o\alpha 1}$) (Jones and Reed, 1987; Hsu et al., 1990) may be activated specifically or with varying degrees of efficiency by cannabinoid receptors, as seen in other receptor systems (McKenzie and Milligan, 1990; Senogles et al., 1990). This could also explain the discrepancies found in our study in the budgerigar brain.

In sum, our data concerning the cannabinoid receptor distribution and cannabinoid-mediated stimulation of G

proteins in the budgerigar brain support the idea that cannabinoid receptors may be involved in a number of roles, such as those described in mammals, such as motor control system, memory tasks, the visual system, or sexual behavior. Discrepancies in the cannabinoid receptor densities and the cannabinoid receptor-mediated stimulation found in several budgerigar brain nuclei are supported by the hypothesis proposed for mammals of the existence of different $G_{i/o}$ protein populations able to associate with cannabinoid receptors, depending on the brain structure, and could reflect the relative weight of cannabinoid transmission in each cerebral area.

4. Experimental procedures

Functional autoradiography was carried out following a protocol previously described for the study of [^{35}S]GTPγS binding in mammals (Sim et al., 1995). Five 1-year-old males were decapitated, and their brains were rapidly removed, frozen in liquid nitrogen and then stored at −80 °C until sectioned. Serial coronal sections of the brain, 10 μm thick, were obtained with a cryostat, mounted on silanized slides and stored at −80 °C for a maximum of 15 days before [^{35}S]GTPγS incubation assays. Every tenth slide was processed for Nissl staining. Slides were thawed at room temperature for 15 min prior to preincubation, followed by 30 min in 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 1 mM DL-dithiothreitol, 0.5% BSA and 2 mM GDP, pH 7.7, at room temperature. Incubations were carried out in the same buffer with 0.05 nM [^{35}S]GTPγS (1250 Ci/mmol; New England Nuclear Corp., Boston, MA, USA), at room temperature for 120 min under four different conditions: (1) incubation buffer (basal [^{35}S]GTPγS binding); (2) incubation buffer in the presence of 10 or 100 μM cannabinoid agonist WIN55,212-2 (total agonist-stimulated [^{35}S]GTPγS binding); (3) incubation buffer in the presence of 10 μM WIN55,212-2 and 10 μM cannabinoid antagonist SR141716A (specificity of cannabinoid-stimulated [^{35}S]GTPγS binding); (4) incubation buffer in the presence of 10 μM GTPγS (non-specific binding). Net [^{35}S]GTPγS stimulation values were calculated as total agonist-stimulated binding minus basal binding values.

Following incubation, the slides were rapidly dipped twice in 50 mM Tris–HCl, pH 7.4, at 4 °C and then washed 15 min twice in the same buffer at 4 °C. Finally, the slides were rapidly dipped in Milli-Q water and dried in a cold air stream overnight.

Sections were exposed to BioMax MR films (Kodak) along with ^{14}C standards for 5 days, after which the films were developed with D-19 to generate autoradiograms that were quantified in a computerized image analysis system (KS300, Kontron). Quantification of the brain nuclei listed in the work was achieved comparing the autoradiographs obtained with Nissl stained sections consecutives to those used for autoradiography and with a budgerigar brain atlas (Roberts et al., 2001).

Quantitative autoradiography was performed following a previously described protocol for mammals (Herkenham et al., 1991). Five 1-year-old male budgerigars were decapitated, and their brains were rapidly removed, frozen in liquid nitrogen, and then stored at −80 °C until sectioned. Coronal brain sections, 10 μm thick, were obtained with a cryostat, mounted on silanized slides, and stored at −80 °C until incubation with the radioligand. Every tenth slide was processed for Nissl staining. The slides were kept at room temperature for 5 min, followed by incubation with 3 nM [^{3}H]CP55,940 (158 Ci/mmol; PerkinElmer Life Science Products, Inc., Boston, MA, USA) in 50 mM Tris–HCl buffer, 5% BSA, pH 7.4 for 2 h at 37 °C. Non-specific binding was defined in the presence of 10 μM WIN55,212-2.

Following incubation, the slides were rapidly dipped twice in 50 mM Tris–HCl, pH 7.4, at 4 °C and then washed twice for 15 min in the same buffer at 4 °C. Finally, the slides were rapidly dipped in Milli-Q water and dried in a cold air stream overnight. Sections were exposed to [^{3}H]Hyperfilm films (Amersham) along with ^{3}H standards for 15 days, and autoradiographs were obtained and quantified as described for receptor autoradiography.

Pearson correlation analyses between receptor densities and net [^{35}S]GTPγS binding values were performed using GraphPad Prism 4 for Windows (GraphPad Prism Software, Inc.).

Nomenclature of the structures studied (Latin Names) (Reiner et al., 2004). Names in italics between brackets indicate the former nomenclature (Karten and Hodos, 1966)

Telencephalon

A (*Ai*) Arcopallium (*Archistriatum intermedium*)
AD (*Aid*) Arcopallium dorsale (*Archistriatum intermedium, pars dorsalis*)
AI (*Aiv*) Arcopallium intermedium (*Archistriatum intermedium, pars ventralis*)
APH Area Parahippocampalis
E Entopallium (*Ectostriatum*)
GP (*PP*) Globus palidus (*Paleostriatum primitivum*)
HA Hyperpallium apicale (*Hyperestriatum accesorium*)
HD Hyperpallium densocellulare (*Hyperestriatum dorsale*)
Hp Hippocampus
L or FL Area L pallii (*Field L*)
M (*HV*) Mesopallium (*Hyperestriatum ventrale*)
N Nidopallium (*Neostriatum*)
NC Nidopallium caudale (*Neostriatum caudale*)
NI Nidopallium intermedium (*Neostriatum intermedium*)
NLC (*NLc*) Nucleus centralis nidopallii lateralis (*Central nucleus of the lateral neostriatum*)
NSTL (*Ac*) Nucleus striae terminalis lateralis (*Nucleus accumbens*)
PB Perientopallial belt (*Periectoestratiatal belt*)
PVt Pallidum ventrale
RA Robust nucleus arcopallialis (*Robust nucleus of the archistriatum*)
TnA (*Tn*) Nucleus taeniae amygdalae (*Nucleus taeniae*)
StL (*PA*) Striatum laterale (*Paleostriatum augmentatum*)
mpStM (*LPO*) Medial part of the striatum mediale (*Lobus paraolfactorius*)
lpStM (*LPO*) Lateral part of the striatum mediale (*Lobus paraolfactorius*)
StMm (*LPOm*) Striatum mediale, pars magnocellularis (*Magnocellular nucleus of LPO*)

Diencephalon

DIP	Nucleus dorsointermedius posterior thalami
DMm	Magnocellular nucleus of the dorsomedial thalamus
DMP	Nucleus dorsomedialis posterior thalami
HL	Nucleus habenularis lateralis
HM	Nucleus habenularis medialis
PMI	Nucleus paramedianus internus thalami
POM	Nucleus preopticus medialis
Rt	Nucleus rotundus
SPC	Nucleus superficialis parvocellularis

Mesencephalon

Ico	Nucleus intercollicularis
Imc	Nucleus isthmi, pars magnocellularis
Ipc	Nucleus isthmi, pars parvocellularis
MLd	Nucleus mesencephalicus lateralis, pars dorsalis
OMd	Nucleus nervi oculomotorii, pars dorsalis
PT	inner area Nucleus pretectalis, inner area
PT	outer area Nucleus pretectalis, outer area
SAC	Stratum album centrale
SGC	Stratum griseum centrale
SGFS	Stratum griseum et fibrosum superficiale
SGP	Stratum griseum periventriculare
SNc (*TPc*)	Substantia nigra, pars compacta (*Nucleus tegmenti-pedunculopontinus*)
SO	Stratum opticum
TO	Tectum opticum

Cerebellum

Gra	Granular layer of the cerebellum
Mol	Molecular layer of the cerebellum

Acknowledgments

This work was supported by grants from the SAF 2002-00292. R. Mostany is a postdoctoral fellow from Fundación Marqués de Valdecilla-IFIMAV.

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