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## Research Report

# Cannabinoid system in the budgerigar brain

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### 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<sub>1</sub> receptor density value (using [<sup>3</sup>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 [<sup>35</sup>S]GTP-γS binding values induced by the selective CB<sub>1</sub> receptor agonist WIN55,212-2 were observed in the nucleus paramedianus internus thalami, and high values of [<sup>35</sup>S]GTP-γS 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<sub>i/o</sub> protein populations able to associate with the cannabinoid receptors, depending on the brain structure, and could reflect the relative importance that cannabinoid transmission could exert in each cerebral area.

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## 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<sub>1</sub> and CB<sub>2</sub>, 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<sub>i/o</sub> proteins (Howlett et al., 1986, 1988).

Although the CB<sub>1</sub> receptor is the predominant subtype in the central nervous system (CNS) in mammals, the presence of the CB<sub>2</sub> subtype has also recently demonstrated (Van Sickle et al., 2005). CB<sub>1</sub> 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 (Rodríguez et al., 2001). Despite the conserved presence of this receptor in the CNS, different patterns of CB<sub>1</sub> receptor distribution have been found between

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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<sub>2</sub> 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<sub>1</sub> receptors seem to be the only type of cannabinoid site in the CNS of birds (Soderstrom and Johnson, 2000), although a CB<sub>2</sub>-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<sub>1</sub> 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<sub>1</sub> 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 [<sup>35</sup>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<sub>1</sub> 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<sub>1</sub> 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 [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding

The highest basal [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding values. See Table 1 and Fig. 1.

### 2.3. Total agonist-stimulated [<sup>35</sup>S]GTPγS binding

Total agonist-stimulated [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding value in this condition was found in the diencephalic PMI nucleus. Very high [<sup>35</sup>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), arcopallium intermedium (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**

	[ <sup>3</sup> H]CP55,940 binding values	Basal [ <sup>35</sup> S]GTPγS binding values	Agonist-induced [ <sup>35</sup> 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	246 ± 5	41 ± 4	275 ± 30
inner area			
PT		185 ± 13	540 ± 55
outer area			
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 [<sup>3</sup>H]CP55,940 are expressed as fmol radioligand/mg equivalent tissue. Basal and agonist-stimulated [<sup>35</sup>S]GTPγS binding values are expressed as nCi/g equivalent tissue.

the molecular layer of the cerebellum (Mol). Moderate [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding values (<25% with respect to the PMI).

#### 2.4. Specificity of cannabinoid-stimulated [<sup>35</sup>S]GTPγS binding

[<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding.

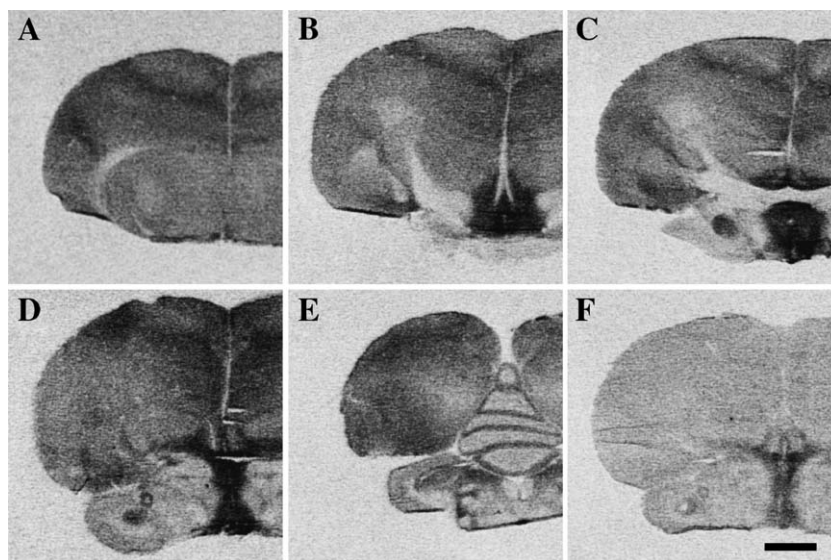
#### 2.5. Receptor autoradiography

The distribution of [<sup>3</sup>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 [<sup>3</sup>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<sub>1</sub> receptor densities (measured as [<sup>3</sup>H]CP55,940 binding values) and CB<sub>1</sub> receptor-mediated stimulation of G proteins (net agonist-stimulated [<sup>35</sup>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 [<sup>35</sup>S]GTPγS/[<sup>3</sup>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 [ $^{35}\text{S}$ ]GTP $\gamma$ S 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<sub>1</sub> 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<sub>i/o</sub> protein stimulation mediated through CB<sub>2</sub> receptors using concentrations of up to 100  $\mu\text{M}$  of this agonist. These results suggest that functional CB<sub>2</sub> 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<sub>2</sub> cannabinoid receptors in the adult chicken brain (Fowler et al., 2001).

The heterogeneous distribution of the [ $^3\text{H}$ ]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 [ $^{35}\text{S}$ ]GTP $\gamma$ S 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

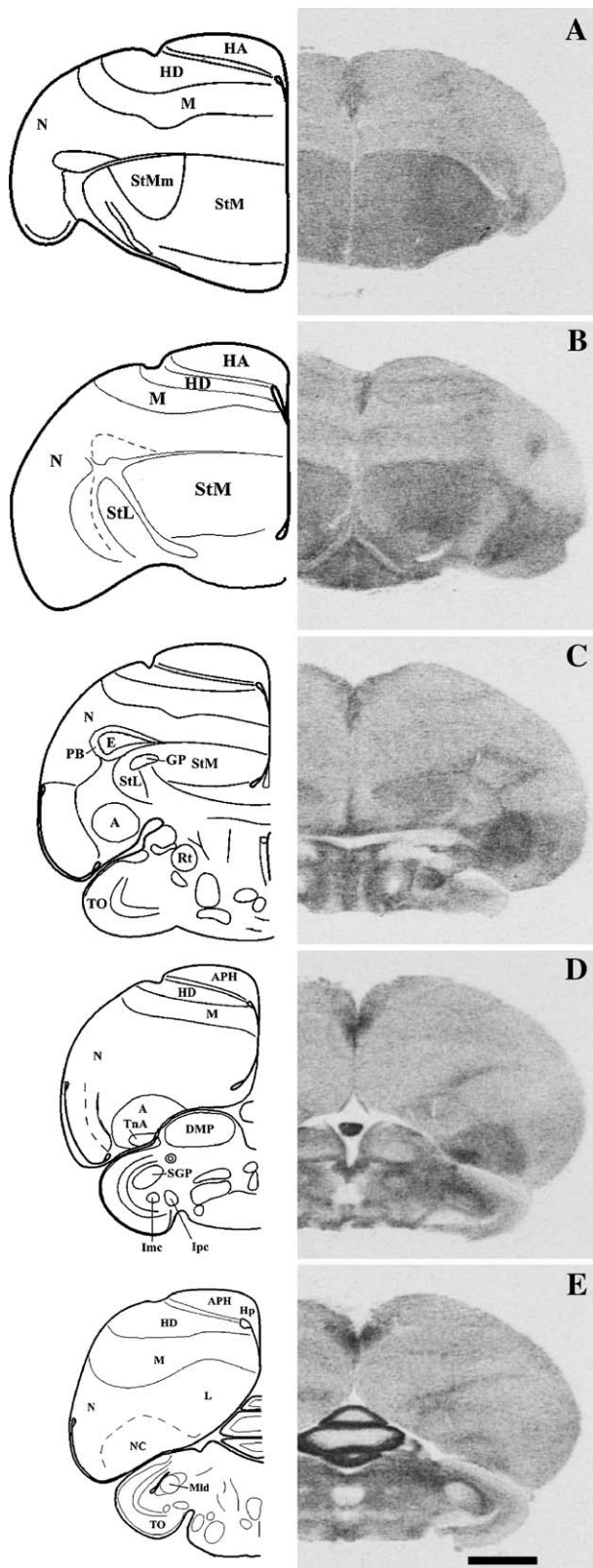
In the cerebellum, very high amounts of CB<sub>1</sub> receptors have been reported in rodents using Western blot assays (Tsou et al., 1998) and “in vivo” autoradiography techniques (Gatley et al.,

1998). Regarding our [ $^3\text{H}$ ]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 [ $^3\text{H}$ ]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  $[^3\text{H}]\text{CP55,940}$  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.**

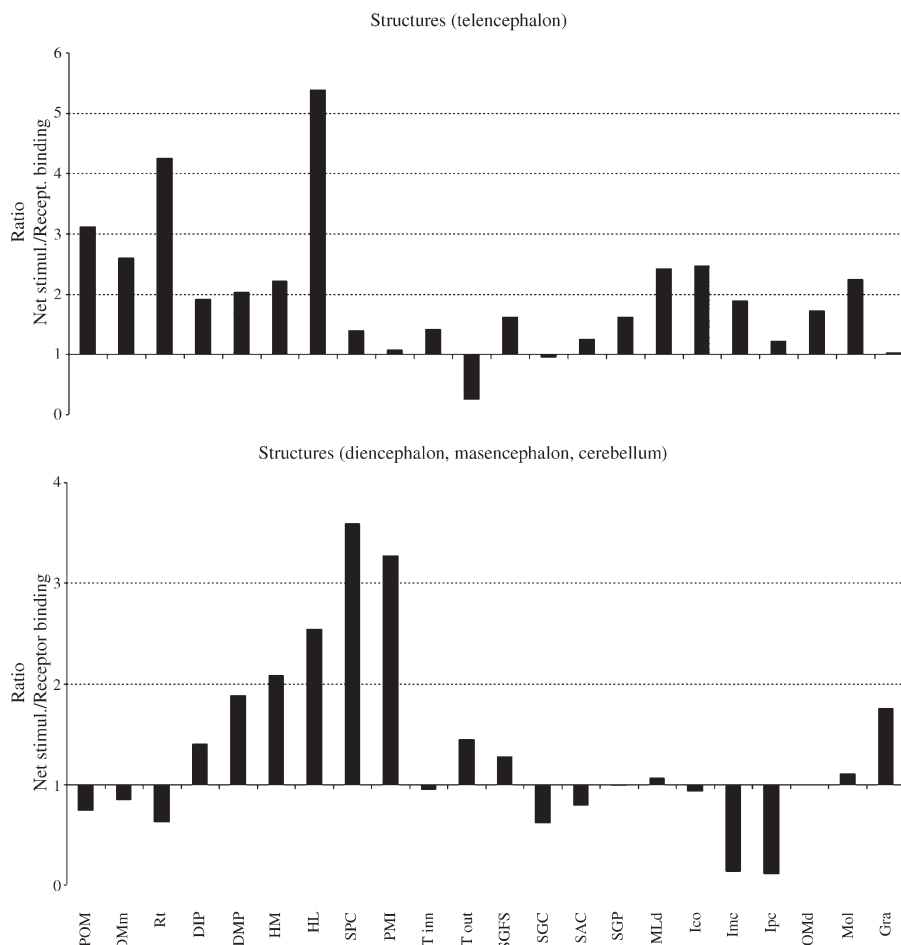
cannabinoid-stimulated  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding values for rat cerebellum and striatum (Breivogel et al., 1997) and higher values in the human basal striatum than in the cerebellum (Rodriguez-Puertas et al., 2000) have been described. On the other hand, our data indicate low 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  $[^3\text{H}]\text{CP55,940}$  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  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  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  $[^3\text{H}]\text{CP55,940}$  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  $[^3\text{H}]\text{CP55,940}$  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 [<sup>35</sup>S]GTP $\gamma$ S mediated by CB receptors (data expressed as nCi/g eq. tissue) and CB receptor binding using [<sup>3</sup>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 [<sup>35</sup>S]GTP $\gamma$ S 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 [<sup>35</sup>S]GTP $\gamma$ S 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<sub>i/o</sub> alpha subunits (G<sub>1</sub> $\alpha$ <sub>1</sub>, G<sub>1</sub> $\alpha$ <sub>2</sub>, G<sub>1</sub> $\alpha$ <sub>3</sub>, G<sub>o</sub> $\alpha$ <sub>1</sub>, G<sub>o</sub> $\alpha$ <sub>1</sub>) (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]GTP $\gamma$ S 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  $-80^{\circ}\text{C}$  until sectioned. Serial coronal sections of the brain,  $10\ \mu\text{m}$  thick, were obtained with a cryostat, mounted on silanized slides and stored at  $-80^{\circ}\text{C}$  for a maximum of 15 days before [ $^{35}$ S]GTP $\gamma$ S 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]GTP $\gamma$ S (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]GTP $\gamma$ S binding); (2) incubation buffer in the presence of 10 or 100  $\mu\text{M}$  cannabinoid agonist WIN55,212-2 (total agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding); (3) incubation buffer in the presence of 10  $\mu\text{M}$  WIN55,212-2 and 10  $\mu\text{M}$  cannabinoid antagonist SR141716A (specificity of cannabinoid-stimulated [ $^{35}$ S]GTP $\gamma$ S binding); (4) incubation buffer in the presence of 10  $\mu\text{M}$  GTP $\gamma$ S (non-specific binding). Net [ $^{35}$ S]GTP $\gamma$ S 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  $4^{\circ}\text{C}$  and then washed 15 min twice in the same buffer at  $4^{\circ}\text{C}$ . 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  $^{14}\text{C}$  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  $-80^{\circ}\text{C}$  until sectioned. Coronal brain sections,  $10\ \mu\text{m}$  thick, were obtained with a cryostat, mounted on silanized slides, and stored at  $-80^{\circ}\text{C}$  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 [ $^3\text{H}$ ]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  $37^{\circ}\text{C}$ . Non-specific binding was defined in the presence of 10  $\mu\text{M}$  WIN55,212-2.

Following incubation, the slides were rapidly dipped twice in 50 mM Tris-HCl, pH 7.4, at  $4^{\circ}\text{C}$  and then washed twice for 15 min in the same buffer at  $4^{\circ}\text{C}$ . Finally, the slides were rapidly dipped in Milli-Q water and dried in a cold air stream overnight. Sections were exposed to [ $^3\text{H}$ ]Hyperfilm films (Amersham) along with  $^3\text{H}$  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]GTP $\gamma$ S 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 ( <i>Archistriatum intermedium</i> )
AD (Aid)	Arcopallium dorsale ( <i>Archistriatum intermedium, pars dorsalis</i> )
AI (Aiv)	Arcopallium intermedium ( <i>Archistriatum intermedium, pars ventralis</i> )
APH	Area Parahippocampalis
E	Entopallium ( <i>Ectostriatum</i> )
GP (PP)	Globus pallidus ( <i>Paleostriatum primitivum</i> )
HA	Hyperpallium apicale ( <i>Hyperestriatum accesorium</i> )
HD	Hyperpallium densocellulare ( <i>Hyperestriatum dorsale</i> )
Hp	Hippocampus
L or FL	Area L pallii ( <i>Field L</i> )
M (HV)	Mesopallium ( <i>Hyperestriatum ventrale</i> )
N	Nidopallium ( <i>Neostriatum</i> )
NC	Nidopallium caudale ( <i>Neostriatum caudale</i> )
NI	Nidopallium intermedium ( <i>Neostriatum intermedium</i> )
NLC (NLC)	Nucleus centralis nidopallii lateralis ( <i>Central nucleus of the lateral neostriatum</i> )
NSTL (Ac)	Nucleus striae terminalis lateralis ( <i>Nucleus accumbens</i> )
PB	Perientopallial belt ( <i>Periectoestratial belt</i> )
PVt	Pallidum ventrale
RA	Robust nucleus arcopallialis ( <i>Robust nucleus of the archistriatum</i> )
TnA (Tn)	Nucleus taeniae amygdalae ( <i>Nucleus taeniae</i> )
StL (PA)	Striatum laterale ( <i>Paleostriatum augmentatum</i> )
mpStM	(LPO) Medial part of the striatum mediale ( <i>Lobus paraolfactorius</i> )
lpStM	(LPO) Lateral part of the striatum mediale ( <i>Lobus paraolfactorius</i> )
StMm	(LPOm) Striatum mediale, pars magnocellularis ( <i>Magnocellular nucleus of LPO</i> )



*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

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## REFERENCES

- Ball, G.F., Hulse, S.H., 1998. Birdsong. *Am. Psychol.* 53, 37–58.
- Balthazart, J., Absil, P., Gérard, M., Appeltants, D., Ball, G.F., 1998. Appetitive and consummatory male sexual behavior in Japanese quail are differentially regulated by subregions of the preoptic medial nucleus. *J. Neurosci.* 18, 6512–6527.
- Berrendero, F., Garcia-Gil, L., Hernandez, M.L., Romero, J., Cebeira, M., de Miguel, R., Ramos, J.A., Fernandez-Ruiz, J.J., 1998. Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. *Development* 125 (16), 3179–3188.
- Brauth, S.E., Liang, W., Amateau, S.K., Robert, T.F., 2005. Sexual dimorphism of vocal control nuclei in budgerigars (*Melopsittacus undulatus*) revealed with Nissl and NADPH-d staining. *J. Comp. Neurol.* 484 (1), 15–27.
- Breivogel, C.S., Sim, L.J., Childers, S.R., 1997. Regional differences in cannabinoid receptor/G-protein coupling in rat brain. *J. Pharmacol. Exp. Ther.* 282 (3), 1632–1642.
- Devane, W.A., Dysarz III, F.A., Johnson, M.R., Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* 34 (5), 605–613.
- Diana, M.A., Levenes, C., Mackie, K., Marty, A., 2002. Short-term retrograde inhibition of GABAergic synaptic currents in rat Purkinje cells is mediated by endogenous cannabinoids. *J. Neurosci.* 22, 200–208.
- Durand, S.E., Heaton, J.T., Amateau, S.K., Brauth, S.E., 1997. Vocal control pathways through the anterior forebrain of a parrot (*Melopsittacus undulatus*). *J. Comp. Neurol.* 377 (2), 179–206.
- Fowler, C.J., Nilsson, O., Andersson, M., Disney, G., Jacobsson, S.O., Tiger, G., 2001. Pharmacological properties of cannabinoid receptors in the avian brain: similarity of rat and chicken cannabinoid1 receptor recognition sites and expression of cannabinoid2 receptor-like immunoreactivity in the embryonic chick brain. *Pharmacol. Toxicol.* 88, 213–222.
- Freund, T.F., Katona, I., Piomelli, D., 2003. Role of endogenous cannabinoids in synaptic signaling. *Physiol. Rev.* 83 (3), 1017–1066.
- Gahr, M., 2000. Neural song control system of hummingbirds: comparison to swifts, vocal learning (Songbirds) and nonlearning (Suboscines) passerines, and vocal learning (Budgerigars) and nonlearning (Dove, owl, gull, quail, chicken) nonpasserines. *J. Comp. Neurol.* 426, 182–196.
- Galiege, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., Casellas, P., 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* 232 (1), 54–61.
- Gatley, S.J., Lan, R., Volkow, N.D., Pappas, N., King, P., Wong, C.T., Gifford, A.N., Pyatt, B., Dewey, S.L., Makriyannis, A., 1998. Imaging the brain marijuana receptor: development of a radioligand that binds to cannabinoid CB1 receptors in vivo. *J. Neurochem.* 70 (1), 417–423.
- Glass, M., Felder, C.C., 1997. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *J. Neurosci.* 17 (14), 5327–5333.
- Herkenham, M., Lynn, A.B., Little, M.D., Johnson, M.R., Melvin, L.S., de Costa, B.R., Rice, K.C., 1990. Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. U. S. A.* 87 (5), 1932–1936.
- Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., de Costa, B.R., Rice, K.C., 1991. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci.* 11 (2), 563–583.
- Hernandez-Tristan, R., Arevalo, C., 1999. Effect of acute delta9-THC treatment on hypothalamic catecholamines and sexual behaviour in male rats. *J. Physiol. Biochem.* 55 (1), 53–54.
- Hile, A.G., Plummer, T.K., Striedter, G.F., 2000. Male vocal imitation produces call convergence during pair bonding in budgerigars. *Melopsittacus undulatus*. *Anim. Behav.* 59, 1209–1218.
- Hoffman, A.F., Lupica, C.R., 2000. Mechanisms of cannabinoid inhibition of GABA(A) synaptic transmission in the hippocampus. *J. Neurosci.* 20 (7), 2470–2479.
- Howlett, A.C., Qualy, J.M., Khachatryan, L.L., 1986. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol. Pharmacol.* 29, 307–313.
- Howlett, A.C., Johnson, M.R., Melvin, L.S., Milne, G.M., 1988. Nonclassical cannabinoid analgesics inhibit adenylate cyclase: development of a cannabinoid receptor model. *Mol. Pharmacol.* 33, 297–302.
- Howlett, A.C., Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E., Porrino, L.J., 2004. Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* 47 (Suppl. 1), 345–358.
- Hsu, W.H., Rudolph, U., Sanford, J., Bertrand, P., Olate, J., Nelson, C., Moss, L.G., Boyd, A.E., Codina, J., Birnbaumer, L., 1990. Molecular cloning of a novel splice variant of the alpha subunit



- of the mammalian Go protein. *J. Biol. Chem.* 265 (19), 11220–11226.
- Iversen, L., 2003. Cannabis and the brain. *Brain* 126 (6), 1252–1270.
- Janata, P., 2001. Quantitative assessment of vocal development in the zebra finch using self-organizing neural networks. *J. Acoust. Soc. Am.* 110, 2593–2603.
- Jarvis, E.D., Mello, C.V., 2000. Molecular mapping of brain areas involved in parrot vocal communication. *J. Comp. Neurol.* 419 (1), 1–31.
- Jarvis, E.D., Ribeiro, S., da Silva, M.L., Ventura, D., Vielliard, J., Mello, C.V., 2000. Behaviourally driven gene expression reveals song nuclei in hummingbird brain. *Nature* 406, 628–632.
- Jones, D.T., Reed, R.R., 1987. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J. Biol. Chem.* 262 (29), 14241–14249.
- Karten, H.J., Hodos, W., 1966. A stereotaxic atlas of the brain of the pigeon (*Columba livia*). In: Karten, H.J., Hodos, W. (Eds.), *The Johns Hopkins Press*, Baltimore, Maryland, USA.
- Levenes, C., Daniel, H., Soubrie, P., Crepel, F., 1998. Cannabinoids decrease excitatory synaptic transmission and impair long-term depression in rat cerebellar Purkinje cells. *J. Physiol.* 510 (Pt. 3), 867–879.
- Maejima, T., Ohno-Shosaku, T., Kano, M., 2001. Endogenous cannabinoid as a retrograde messenger from depolarized postsynaptic neurons to presynaptic terminals. *Neurosci. Res.* 40 (3), 205–210.
- Mailleux, P., Vanderhaeghen, J.J., 1992a. Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. *Neuroscience* 48 (3), 655–668.
- Mailleux, P., Vanderhaeghen, J.J., 1992b. Localization of cannabinoid receptor in the human developing and adult basal ganglia. Higher levels in the striatonigral neurons. *Neurosci. Lett.* 148 (1–2), 173–176.
- Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346 (6284), 561–564.
- McKenzie, F.R., Milligan, G., 1990. Delta-opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein Gi2. *Biochem. J.* 267 (2), 391–398.
- Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365 (6441), 61–65.
- Piomelli, D., Giuffrida, A., Calignano, A., Rodriguez de Fonseca, F., 2000. The endocannabinoid system as a target for therapeutic drugs. *Trends Pharmacol. Sci.* 21, 218–224.
- Plummer, T.K., Striedter, G.F., 2000. Auditory responses in the vocal motor system of budgerigars. *J. Neurobiol.* 142, 79–94.
- Reiner, A., Perkel, D.J., Bruce, L.L., Butler, A.B., Csillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., Striedter, G., Wild, M., Ball, G.F., Durand, S., Gunturkun, O., Lee, D.W., Mello, C.V., Powers, A., White, S.A., Hough, G., Kubikova, L., Smulders, T.V., Wada, K., Dugas-Ford, J., Husband, S., Yamamoto, K., Yu, J., Siang, C., Jarvis, E.D., 2004. Revised nomenclature for avian telencephalon and some related brainstem nuclei. *J. Comp. Neurol.* 473 (3), 377–414.
- Roberts, T.F., Cookson, K.K., Heaton, K.J., Hall, W.S., Brauth, S.E., 2001. Distribution of tyrosine hydroxylase-containing neurons and fibers in the brain of the budgerigar (*Melopsittacus undulatus*): general patterns and labeling in vocal control nuclei. *J. Comp. Neurol.* 429, 436–454.
- Rodriguez, J.J., Mackie, K., Pickel, V.M., 2001. Ultrastructural localization of the CB<sub>1</sub> cannabinoid receptor in mu-opioid receptor patches of the rat caudate putamen nucleus. *J. Neurosci.* 21 (3), 823–833.
- Rodriguez-Puertas, R., Gonzalez-Maeso, J., Meana, J.J., Pazos, A., 2000. Autoradiography of receptor-activated G-proteins in post mortem human brain. *Neuroscience* 96, 169–180.
- Senogles, S.E., Spiegel, A.M., Padrell, E., Iyengar, R., Caron, M.G., 1990. Specificity of receptor-G protein interactions. Discrimination of Gi subtypes by the D2 dopamine receptor in a reconstituted system. *J. Biol. Chem.* 265 (8), 4507–4514.
- Sim, L.J., Selley, D.E., Childers, S.R., 1995. In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5-[gamma-[<sup>35</sup>S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. U. S. A.* 92, 7242–7246.
- Soderstrom, K., Johnson, F., 2000. CB1 cannabinoid receptor expression in brain regions associated with zebra finch song control. *Brain Res.* 857 (1–2), 151–157.
- Soderstrom, K., Johnson, F., 2003. Cannabinoid exposure alters learning of zebra finch vocal patterns. *Brain Res. Dev. Brain Res.* 142 (2), 215–217.
- Soderstrom, K., Tian, Q., 2004. Distinct periods of cannabinoid sensitivity during zebra finch vocal development. *Brain Res. Dev. Brain Res.* 153 (2), 225–232.
- Striedter, G.F., 1994. The vocal control pathways in budgerigars differ from those in songbirds. *J. Comp. Neurol.* 343, 35–36.
- Theiss, M.P., Hellmann, B., Gunturkun, O., 2003. The architecture of an inhibitory sidepath within the avian tectofugal system. *NeuroReport* 14 (6), 879–882.
- Tsou, K., Brown, S., Sanudo-Pena, M.C., Mackie, K., Walker, J.M., 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83 (2), 393–411.
- Van Sickle, M.D., Duncan, M., Kingsley, P.J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J.S., Marnett, L.J., Di Marzo, V., Pittman, Q.J., Patel, K.D., Sharkey, K.A., 2005. Identification and functional characterization of brainstem cannabinoid CB<sub>2</sub> receptors. *Science* 310 (5746), 329–332.