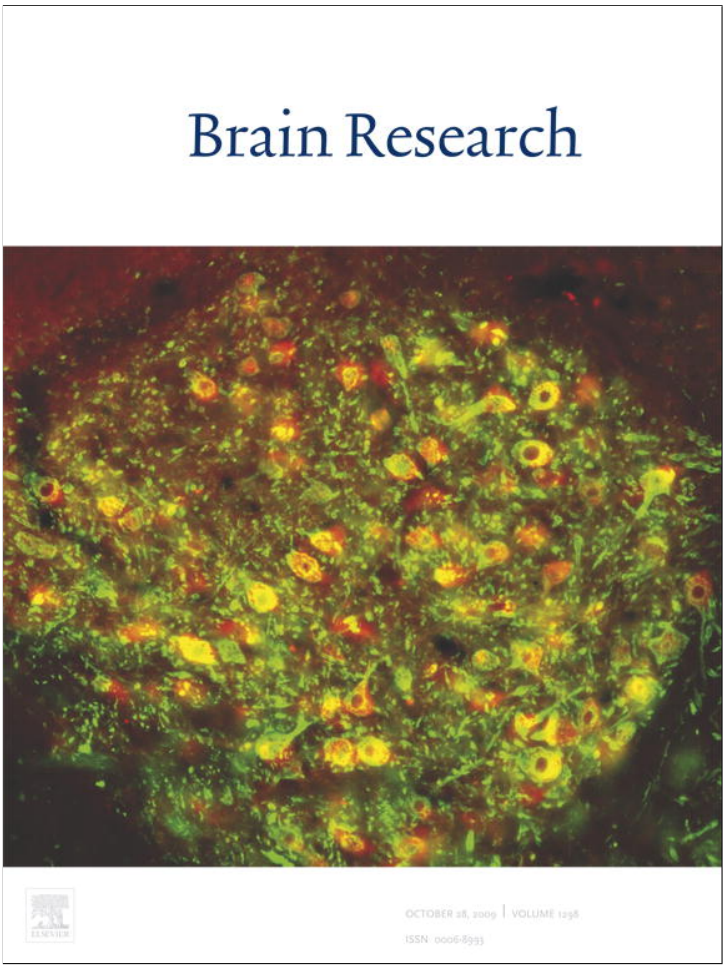

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Research Report

Identification of calcium sensing receptor (CaSR) mRNA-expressing cells in normal and injured rat brain

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ABSTRACT

Calcium sensing receptor (CaSR), isolated for the first time from bovine and human parathyroid, is a G-protein-coupled receptors that has been involved in diverse physiological functions. At present a complete in vivo work on the identification of CaSR mRNA-expressing cells in the adult brain lacks and this investigation was undertaken in order to acquire more information on cell type expressing CaSR mRNA in the rat brain and to analyse for the first time its expression in different experimental models of brain injury. The expression of CaSR mRNAs was found mainly in scattered cells throughout almost all the brain regions. A double labeling analysis showed a colocalization of CaSR mRNA expression in neurons and oligodendrocytes, whereas it was not found expressed both in the microglia and in astrocytes. One week after kainate-induced seizure CaSR was found in the injured CA3 region of the hippocampus and very interestingly it was found up-regulated in the neurons of CA1–CA2 and dentate gyrus. Similarly, 1 week following ibotenic acid injection in the hippocampus, CaSR mRNA expression was increased in oligodendrocytes both in the lesioned area and in the contralateral CA1–CA3 pyramidal cell layers and dentate gyrus. One week after needle-induced mechanical lesion an increase of labeled cells expressing CaSR mRNA was observed along the needle track. In conclusion, the present results contribute to extend available data on cell type-expressing CaSR in normal and injured brain and could spur to understand the role of CaSR in repairing processes of brain injury.

1. Introduction

Calcium sensing receptor (CaSR) has been isolated for the first time from bovine and human parathyroid glands (Brown, 1991; Garrett et al., 1995), and later from rat brain (Ruat et al., 1995). The CaSR is predicted to be glycosylated proteins, with a large extracellular domain and the seven membrane spanning region characteristic of other G-protein-coupled receptors (Brown, 1991; Garrett et al., 1995; Riccardi et al., 1995), and it has been involved in diverse physiological functions (Chattopadhyay and Brown, 2000). Localization of transcripts for CaSR in the adult rat brain has been reported by Rogers KV and

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Abbreviations: CaSR, Calcium sensing receptor; mGluRs, metabotropic glutamate receptors

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Table 1 – Distribution of CaSR mRNA in the adult rat brain.

Brain regions	Density of labeled cells
Olfactory bulb	
Internal plexiform layer	+
Anterior olfactory nucleus medial and posterior part	++
Ventral part	+++
Tenia tecta	+++
Cerebral cortex	+
Hippocampal formation	
CA1 and CA3 fields of Ammon's horn	+
CA2 field of Ammon's horn	+++
Stratum Oriens and stratum radiatum hippocampus	+
Subiculum	++
Septum	
Medial septal nucleus	+
Lateral septal nucleus	+
Septohippocampal nucleus	++
Septofimbrial nucleus	+
Septohippocampal nucleus	++
Nucleus of the horizontal limb diagonal band	++
Basal ganglia	
Caudate–putamen	+
Globus pallidus	+
Amygdala	
Amygdaloid complex	+
Amygdalohippocampal area	+++
Thalamus	
Ventrolateral thalamic nucleus	+
Ventral posteromedial thal. nu.	+
Ventral posterolateral thal. nu.	+
Habenular nuclei	+
Hypothalamus	
Paraventricular hypoth. nu., medial parvicell.	++
Anterior medial preoptic nu.	++
Medial preoptic nu.	++
subfornical organ	++++
Arcuate hypoth nu.	++
Lateral hypothalamic area	+
Dorsal hypoth area	++
Dorsomedial hypoth. nu.	+
Dorsomedial hypoth. nu. compact	++
Premammillary nu., ventral part	+
Lateral mammillary nu.	++
Tuberomammillary nu.ventral part	++
Periventricular hypoth. nu.	++
Mammillothalamic tract	++
Suprachiamatic and supraoptic nu.	+
Tuber cinereum area	++
Ventromedial hypoth. Nu	+
Mesencephalon	+
Interpeduncular nu.	+
Geniculate nu.	+
Inferior and superior colliculus	+
Central gray	+
Cerebellum	
Cerebellar cortex	+
Cerebellar nu.	+
Brain stem	
Several nuclei and fibers tract	+
Dorsal nu. of lateral lemniscus	+++
Dorsal tegmental bundle	++
Area postrema	++
White matter	
Forceps major	++
Forceps minor	++
Cingulum	+
Fimbria hippocampus	++
Fornix	+
Stria terminalis	++
Internal and external capsule	+
Medial forebrain bundle	++
Commissure	+
Corpus callosum	++
Dorsal root ganglia	+++
Spinal cord	+

Relative density of CaSR mRNA expressing cells: (+) low, (++) moderate, (+++) high, (++++) highest. The data are shown using an arbitrary semiquantitative scale of labeling density described as "low density of labeling" (+), indicating the percentage of labeled cells between 5 and 20%; as "moderate density of labeling" (++), indicating the percentage of labeled cells between 20 and 40 %; and as "high density of labeling" (+++), indicating the percentage of labeled between 40 and 80 %; and "highest density of labeling" (++++) indicating the percentage of labeled between 80 and 100%.

coworkers (1997), and more recently by Ferry et al. (2000), which performed an analysis of CaSR mRNA expression in the adult brain and its localization in neurons and oligodendrocytes. CaSR has been also found express both in astrocytes and microglia but these results have been achieved using primary cultures of human and rat brain respectively (Chattopadhyay et al., 1999, 2000). Therefore, at present, a complete in vivo work on the identification of CaSR mRNA-expressing cells lacks and this study was undertaken in order to acquire more information on cell type expressing CaSR mRNA in the rat brain and to analyse for the first time the CaSR mRNA expression in different experimental models of brain injury: (a) bilateral lesion of hippocampal CA3 regions by kainate-induced seizure; (b) unilateral hippocampal lesion by a stereotaxic local injection of ibotenic acid; (c) mechanical lesion produced by a needle (30 G) stereotaxically introduced through the rostral cerebral cortex.

2. Results

2.1. *Expression of CaSR mRNA*

A detailed analysis of CaSR expression in the normal rat brain was performed using in situ hybridization. Cells expressing CaSR mRNA were scattered throughout almost all the brain

areas with regional differences in labeled cells density. Brain regions and nuclei that express CaSR mRNAs are listed in Table 1, that report the relative density of CaSR-expressing cells ranked on a scale from one to four, with "+" representing widely scattered cells (5-20%) and "++++" representing the highest cell density (80–100%). Figs. 1–3 show representative brain regions and nuclei expressing CaSR mRNA. The specificity of CaSR in situ hybridization assays was determined by the use of a sense strand control probe (Fig. 4) and by using as positive control the thyroid tissue in which CaSR mRNA is selectively expressed in the parafollicular C cells (data not shown).

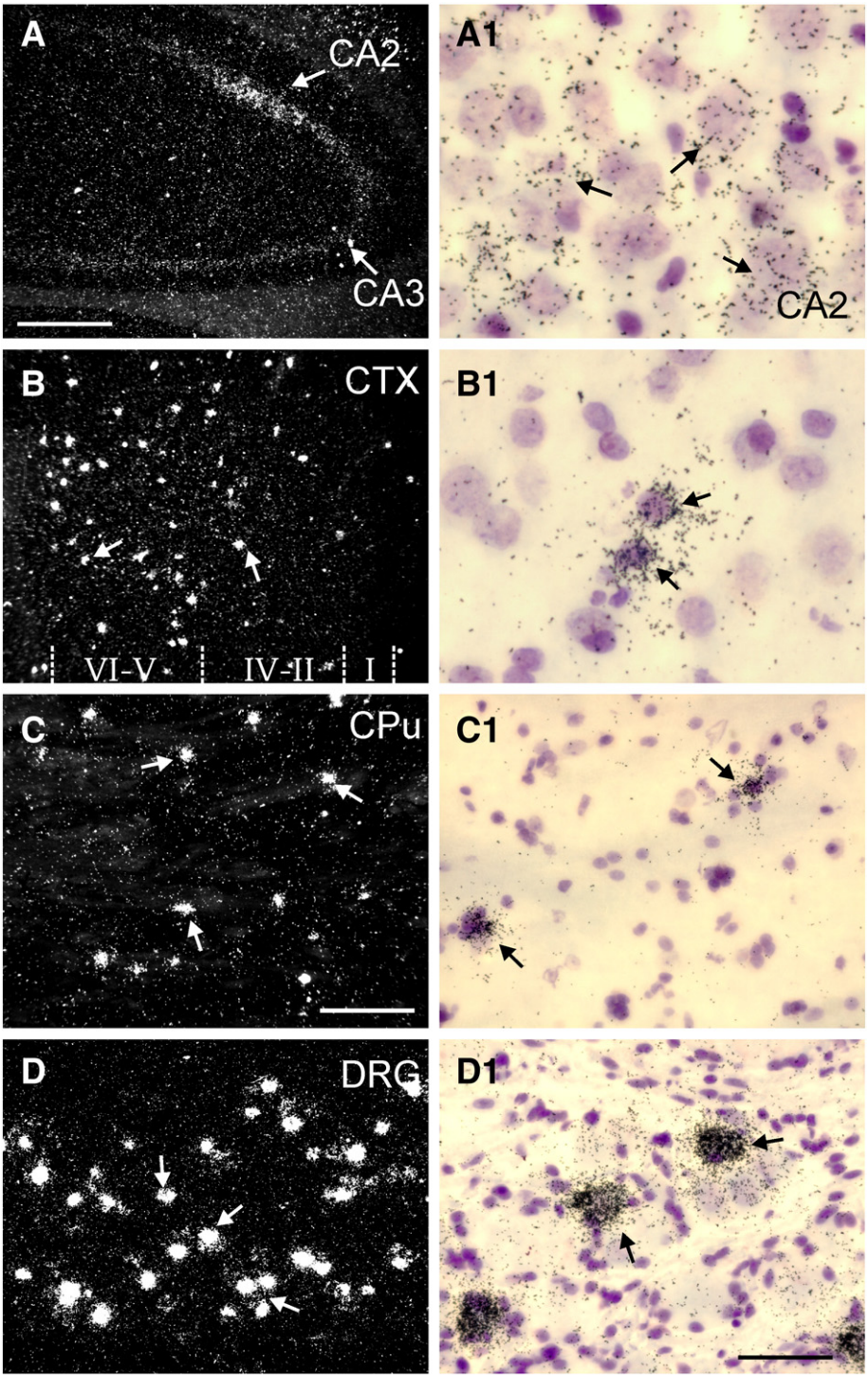

Fig. 1 – Autoradiophotograms in dark (A–D) and bright (A1–D1) field showing the CaSR mRNA expression in various brain regions: CA2 and CA3 pyramidal layers of the hippocampal region (A–A1); CTX, cerebral cortex (B–B1); CPu, caudate–putamen (C–C1); DRG, dorsal root ganglia (D–D1); Arrows indicate cells expressing CaSR mRNA. Scale bar: (A, B) 300 μm; (C, D) 200 μm; (A1–B1) 25 μm; (C1–D1) 50 μm.

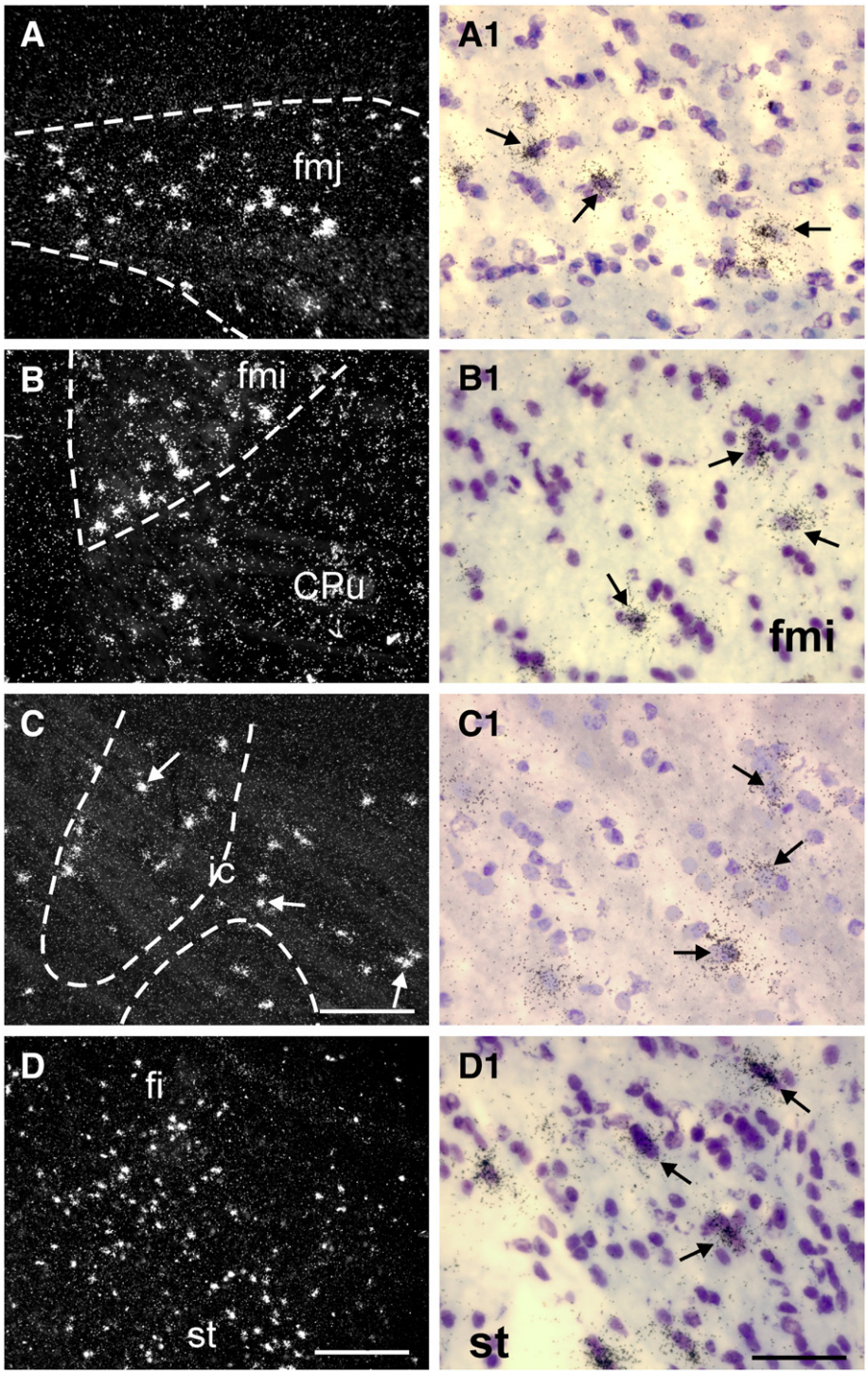

Fig. 2 – Autoradiophotograms in dark (A–D) and bright (A1–D1) field showing the CaSR mRNA expression in various brain regions riches in neuronal fibers: fmj, forceps major of the corpus callosum (A-A1); fmi, forceps minor of the corpus callosum (B–B1); ic, internal capsule (C–C1); fi and st, fimbria of the hippocampus and stria terminalis respectively (D–D1). CPu, caudate–putamen. Arrows indicate cells expressing CaSR mRNA. Scale bar: (A–C) 200 μm; D 300 μm (A1–C1) 50 μm.

2.2. *Colocalization of CaSR mRNA in neurons and glial cells*

The double labeling analysis by in situ hybridization for CaSR mRNA and immunohistochemistry for specific cellular markers (NeuN, GFAP, CNPase and CD11b), allowed us to identify the cell types expressing CaSR. Neurons, identified as NeuN-positive cells, show CaSR expression in several brain regions (Fig. 5). In all the brain regions examined, the double labeling analysis shows a localization

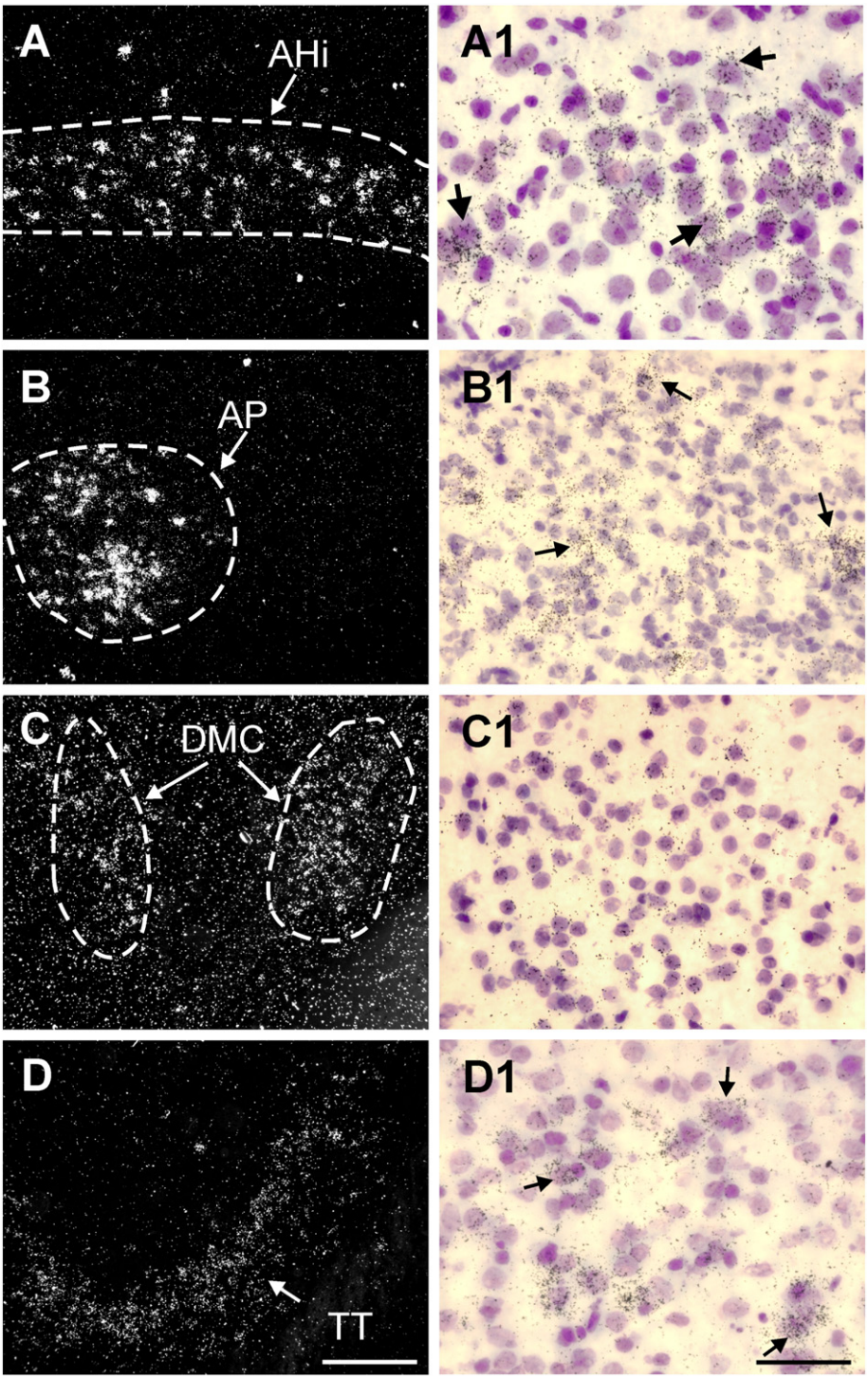

Fig. 3 – Autoradiophotograms in dark (A–D) and bright (A1–D1) field showing the CaSR mRNA expression in various brain nuclei: AHi, anterior hypothalamic area (A–A1); AP, area postrema (B–B1); DMC, dorsal hypothalamic nucleus, compacta part (C–C1); TT, tenia tecta (D–D1). Arrows indicate cells expressing CaSR mRNA. Scale bar: (A–D) 200 μm; (A1–D1) 50 μm.

of CaSR mRNA in oligodendrocytes, identified as CNPase-positive cells (Fig. 6). By contrast, CaSR mRNA is not expressed both in the microglia, identified as CD11b-positive cells and in astrocytes, identified as GFAP-positive cells (Fig. 6).

2.3. *Expression of CaSR mRNA following brain lesion*

To define the regulation of CaSR mRNA expression in response to brain injury an in situ hybridization study was performed at different time points (24 h, 48 h, 72 h and 1 week). We used

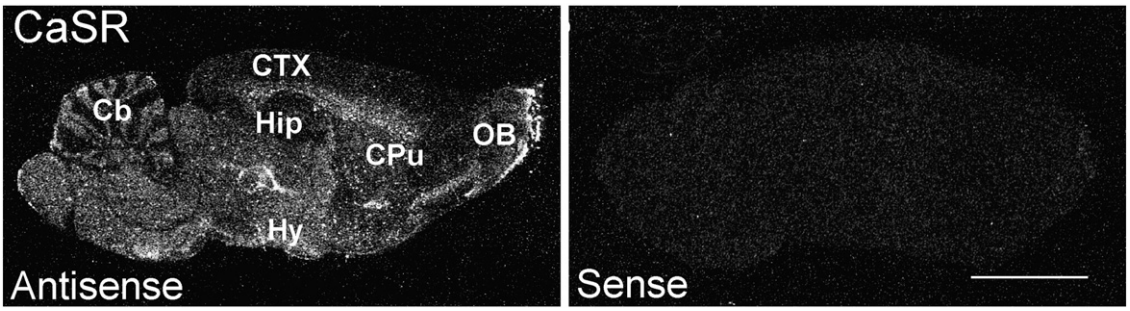

Fig. 4 – Photomicrograph from film autoradiogram of CaSR mRNA expression in the adult rat brain. Representative view of sagittal brain section at lateral level L 2.40 mm, according to Paxinos and Watson Atlas. CTX, cerebral cortex; Cpu, caudate–putamen; Hip, hippocampus; Hy, hypothalamus; OB, olfactory bulbs; Cb, cerebellum; Sense, brain section hybridized with sense probe. Scale bar, 5 mm.

three different experimental brain lesions: Kainate-induced seizure, Ibotenic acid-induced lesion and mechanical lesion induced by needle insertion.

2.3.1. *Kainate-induced seizure*

The seizure induced by intracerebroventricular kainic acid injection produced a neuronal death mainly in the CA3 pyramidal layer of the hippocampus and consequently an activation of gliosis in this hippocampal region (Belluardo et al., 1996). At early post-injury times (24, 48, 72 h) no changes in CaSR expression were detected. At 1 week post-injury two different phenomena were observed (Fig. 7 and Table 2): (1) the appearance of several labeled non-neuronal cells in the injured CA3 region; (2) an increase of the amount of hybridization grains per cells and number of labeled cells in the CA1–CA3 subfield and in the dentate gyrus of hippocampal formation.

2.3.2. *Ibotenic acid-induced hippocampal injury*

We also examined the CaSR mRNA expression at different time points after unilateral injection in the dorsal hippocampal formation of ibotenic acid, a neurotoxin with excitotoxic properties (Condorelli et al., 1988). In the lesioned area we observed an increased number of non-neuronal CaSR mRNA expressing cells 1week following ibotenic acid injection (Fig. 7 and Table 2). These cells were localized both inside and along the border of the lesioned area and showed a scattered distribution (Fig. 7). Interestingly, 1 week after unilateral injection of ibotenic acid, an increased CaSR expression was also observed in the contralateral CA1–CA3 pyramidal cell layers and in the dentate gyrus involving both the percentage of labeled cells and the grain density per cell (Fig. 7 and Table 2).

2.3.3. *Mechanical injury in the cerebral cortex and in the corpus callosum*

We performed a mechanical lesion by insertion of a needle in the cerebral cortex and in the underlying corpus callosum at the rostral caudate–putamen level. In the control rat, the CaSR mRNA was expressed in scattered cells both in the cerebral cortex and in the corpus callosum. One week after mechanical lesion an increased density of labeled cells expressing CaSR mRNA was observed along the needle track both in the cerebral cortex and in the corpus callosum (Fig. 7 and Table 2).

2.4. *Identification of CaSR mRNA expressing cells in the area of lesion*

To identify the non-neuronal cell type expressing CaSR mRNA in the injured area we performed a double labeling analysis using specific markers for glial cells. This analysis showed, in all experimental models of brain lesion used, a localization of CaSR mRNA in CNPase-positive cells. Representative images of this result in ibotenic acid-induced lesion are shown in Fig. 8.

3. Discussion

Results regarding the brain distribution of CaSR transcripts are in agreement with data of previous works (Ferry et al., 2000; Rogers et al., 1997) confirming an expression of CaSR in all brain regions, with a particularly high abundance in subfornical organ, olfactory bulb and hypothalamus. However, we observed different intensity of signal in some brain regions or nuclei (e.g. hippocampal layers, hypothalamus nuclei) in comparison to previously reported data (Rogers et al. 1997). Such minor discrepancies might be related to the lower length of riboprobe (627 bp) used in the present work as compared to that (1.2 kb) used by Rogers et al. (1997) or to in situ hybridization procedure efficacy. A feature of cells expressing CaSR mRNA was their scattered distribution in mostly brain regions, suggesting the possibility that this receptor is expressed in a neuronal or oligodendroglial network with a key role for extracellular calcium sensing. This could be the case of CaSR expression in the white matter for oligodendroglial cells or in the cerebral cortex and in the striatum for neurons. By contrast, some brain nuclei showed very high levels of CaSR mRNA suggesting a role of CaSR in region-specific neuronal functions, such as in the subfornical organ for the regulation of ionic calcium levels in the blood (Sibbald et al., 1988) or in the area postrema for autonomic control of several physiological systems, including the cardiovascular system and the systems controlling feeding and metabolism (Fry and Ferguson, 2007). However, the CaSR present in neuronal cells could potentially contribute to the control of Ca^{+}_{2} levels, which in turn are involved in a variety of neuronal functions, such as signal transduction, generation

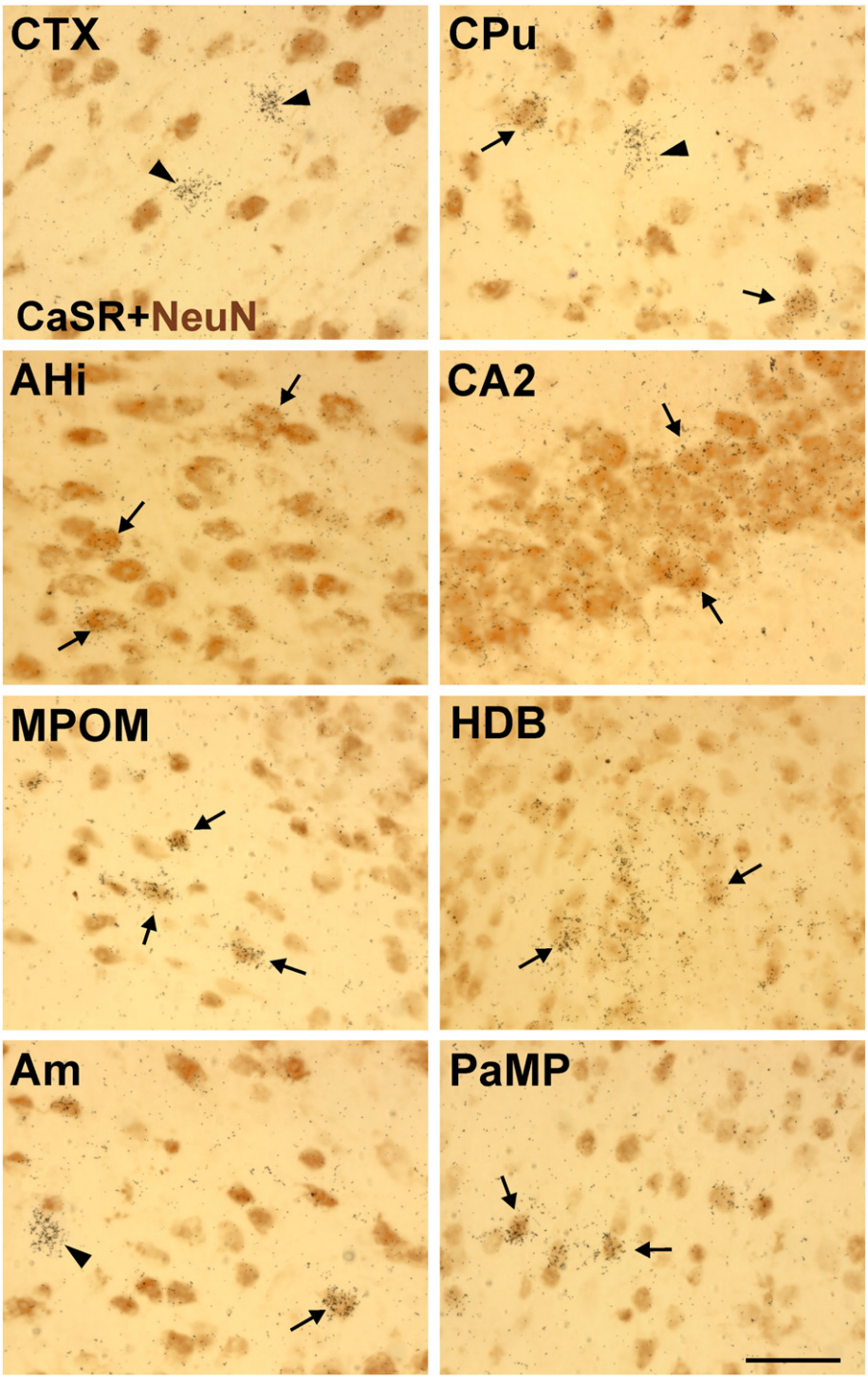

Fig. 5 – Analysis of cell type-expressing CaSR mRNA in various brain regions. Autoradiophotograms from coronal sections showing in high magnification that CaSR mRNAs colocalize with the neuronal marker NeuN. CTX, cerebral cortex; CPu, caudate–putamen; AHi, Anterior hypothalamic area; CA2 pyramidal layer of the hippocampus; MPOM, medial preoptic nucleus medial part; HDB, nucleus of the horizontal limb of diagonal band; Am, amygdala nucleus; PaMP, paraventricular hypothalamic nucleus, medial parvicellular part. Arrows indicate CaSR/NeuN double labeled cells. Arrowhead indicate clusters of CaSR mRNA grains that did not colocalize with NeuN. Scale bar: 50 μm.

and propagation of action potentials, and neurotransmitter release. On this regard, immunohistochemistry has revealed a localization of CaSR within nerve terminals, suggesting that CaSR may regulate neurotransmitter synthesis or release in response to Ca^{+}_{2} levels in the synaptic space and therefore may modulate synaptic responsiveness and plasticity (Jinno et al., 2002; Ruat et al., 1995; Rusakov and Fine, 2003; Vassilev et al., 1997).

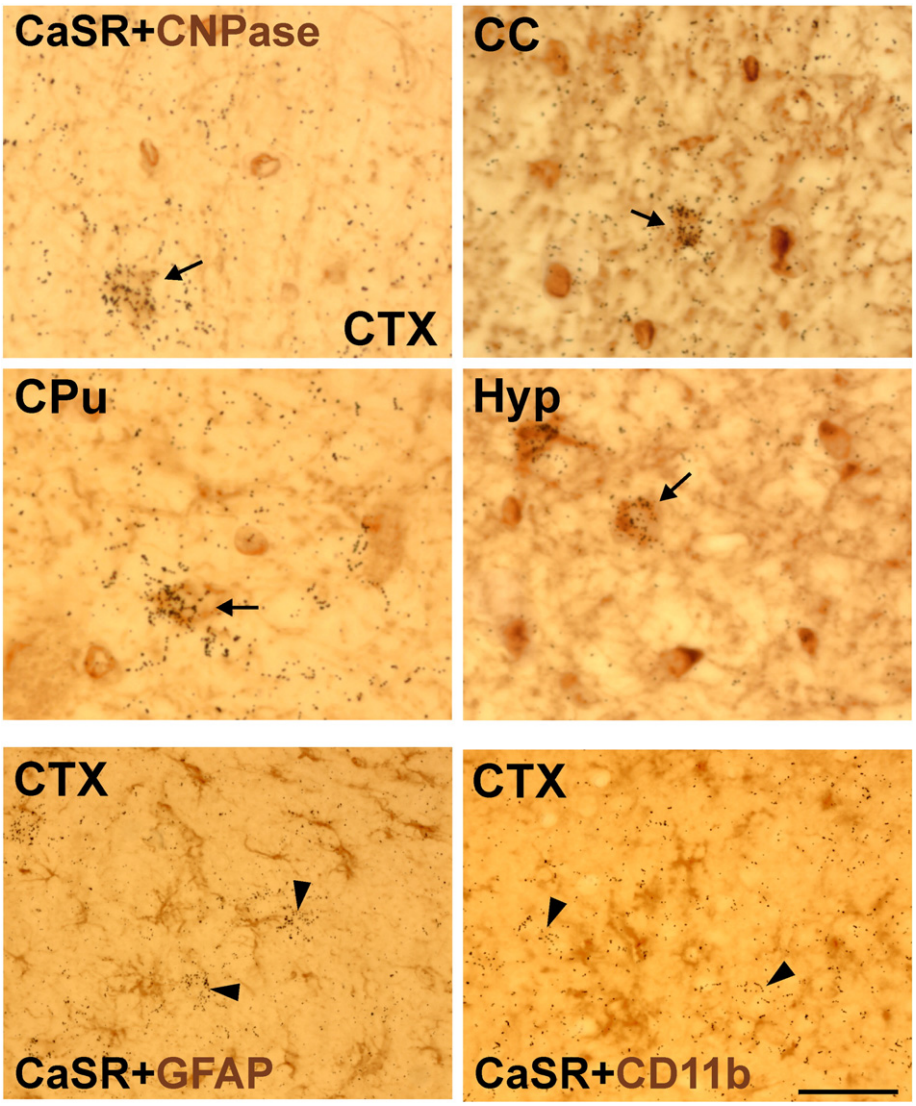

Fig. 6 – Analysis of cell type-expressing CaSR mRNA in various brain regions. Autoradiophotograms from coronal sections showing in high magnification that CaSR mRNAs colocalize with oligodendroglial marker CNPase: CTX, cerebral cortex; cc, corpus callosum; CPu, caudate–putamen; Hyp, hypothalamus. In contrast, CaSR mRNA does not colocalize with GFAP and CD11b markers for astrocytes and microglia, respectively, as shown in representative sections of cerebral cortex (CTX). Arrows indicate CaSR/CNPase double labeled cells. Arrowhead indicate clusters of CaSR mRNA grains that does not colocalize with GFAP and CD11b markers. Scale bar: 50 μm.

The main goal of present work was to identify CaSR mRNA-expressing cells in the rat brain and to study the effects of brain injury on CaSR mRNA expression. Using a double labeling method, we could confirm that CaSR is expressed both in neuronal and non-neuronal cells and among the latters we could show that CaSR is expressed in oligodendrocytes, but not in microglia and astrocytes. However, in a given brain region not all oligodendrocytes express CaSR mRNA, suggesting that this receptor is localized in a subset of these cells. In the present work, CaSR expression in the oligodendrocytes is in agreement with previous data (Ferry et al., 2000), whereas the absence of CaSR in astrocytes and microglia is not (Chattopadhyay et al., 1999, 2000). It is noteworthy that although in an earlier study Chattopadhyay and Brown (2000) failed to detect expression of the CaSR in primary culture of astrocytes isolated from rat brain, later these and others authors demonstrated the presence of CaSR mRNA and protein in primary human astrocytes (Chattopadhyay et al., 2000; Dal Pra et al., 2005). The function of the CaSR in normal oligodendroglial cells is currently unknown, but it is reasonable to hypothesize a role in sensing and regulating local ionic homeostasy during neuronal activity. CaSR has been linked to modulation of the activities of at least two types of ion channels, non selective cation channels and Ca^{+}_{2}-activated K^{+} channels, and glial cells may control neuronal excitability by regulating extracellular K^{+} (Vassilev et al., 1997). The CaSR present in oligodendroglial cells could potentially contribute to other functions since regulation of the extracellular calcium concentration is crucial for numerous cellular processes that include maintenance of membrane potential and control of cellular proliferation,

differentiation and secretion (Brown and MacLeod, 2001; Chattopadhyay et al., 1998).

Although the expression of CaSR has largely been studied in normal condition, no data have been reported for CaSR expression in injured brain. Using three models of brain lesion we could detect in the injured area an increase of CaSR mRNA-expressing cells, that have been identified as oligodendrocytes and not as astrocytes or microglia. A feature of CaSR expression following brain injury was its delayed appearance (7 days post-lesion) in the area of damage, even if the glial reaction (reactive gliosis) can be already observed after two days. In addition, in the area of gliosis not all the CNPase-positive oligodendroglial cells express CaSR mRNA, suggesting the involvement of a subset of oligodendrocytes in the function of Ca_2^+ sensing cells. The delayed response of CaSR expression to brain injury could be related to a local extracellular environmental signals with a critical role in the gliotic tissue organization. In addition to the glial expression in the damaged area, we observed also a clear increase in the level of CaSR mRNA in neuronal cells. The delayed up-regulation of CaSR mRNA in the CA1–CA2 and the dentate gyrus of hippocampal formation following seizure-induced by kainate treatment could depend on modification of neuronal activity or on reduced levels of Ca_2^+ in the local microenvironment. According with this possibility, in some brain conditions characterized by increased neuronal activity and in certain pathological states such as seizures, ischemia, and hypoglycemia, Ca_2^+ levels within the extracellular fluid of the brain are reduced and could potentially be sensed by CaSR (Heinemann et al., 1977).

CaSR displays significant homology with metabotropic glutamate receptors (mGluRs) expressed in the brain (Nakanishi, 1992; Ruat et al., 1995), and it has been shown that some types of mGluRs, including mGluR3 and mGluR5, are activated not only by glutamate but also by extracellular Ca_2^+ (Kubo et al., 1998), although the physiological relevance of this Ca_2^+-sensing property remains unknown. Interesting, similarly to CaSR, in a previous work we found an increased expression of mGluR3 and mGluR5 one week after ibotenic acid or kainate-induced lesions in the rat brain. Such expression changes were localized in oligodendroglial cells or in neurons of dentate gyrus and CA1 pyramidal cell layer (Mudo et al., 2007). Therefore, Ca_2^+ sensing ability by both mGluRs and CaSR in the area of lesion suggests a relevant role of extracellular Ca_2^+ for regulation of cellular processes in response to brain damage (Verkhratsky and Kettenmann, 1996).

In conclusion, the present results contribute to extend available data on CaSR expressing cell types in normal and injured brain and spur-renewed interest on the role of CaSR and other calcium sensing receptors in repairing processes after brain injury.

4. Experimental procedures

Wistar adult male rats (250 g b.w.) from local stock have been used for the present study. The rats were kept under controlled temperature and standardized lighting and free access to food and water. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies. All efforts were made to minimize the number of animals used and their suffering and all experiments were approved by the local ethical committee

4.1. *Brain lesion*

The CaSR mRNA expression was studied in three models of brain injury leading to reactive gliosis: (1) ibotenic acid injection in the dorsal hippocampus as described in Condorelli et al. (1988); (2) epilepsy induced by kainic acid injection in the lateral ventricle as described in Mudò et al. (1995); (3) mechanical lesion produced by a needle (30 G) stereotaxically introduced through the rostral cerebral cortex until it entered in the striatum for 1 mm as described in Cheng et al. (2008). Ibotenic acid (Sigma Chem. Co St. Louis, MO) was dissolved in phosphate buffered saline (PBS), 0.05 M final concentration, and pH adjusted to 7.2 with NaOH and injected at a volume of 0.5 μl in the right dorsal hippocampus. Rats treated with kainic acid (Sigma, St. Louis, MO) were injected bilaterally in the brain lateral ventricle (0.35 μg/0.5 μl PBS). For the lesions, the rats were anesthetized with chloral hydrate and a David Kopf stereotaxic apparatus was used for drug injection and mechanical lesion according the following coordinates (Paxinos and Watson, 1999): for ibotenic acid injection, Bregma: AP 4.16 mm, V 4.2 mm, L 1.9 mm; for kainic acid injection, Bregma: AP +0.2, L 1.5, V 4.2; for mechanical lesion Bregma: AP, +1.20 mm; V, 4 mm; L, 1.5 mm. At different time from the brain lesion (24 h, 48 h, 72 h and 1 week) the rats (three for each group) were killed by decapitation under deep anesthesia and brains were frozen in cooled isopentane and stored at −70 °C until use.

4.2. *In situ hybridization*

In the present study we examined by in situ hybridization the gene expression of CaSR in both normal condition and following brain injury.

Fig. 7 – Autoradiophotograms in dark field (A–D) showing CaSR mRNA expression in the hippocampal formation in the following condition: in control rat (A) and in rat 1 week after injection of kainate-induced seizures (B); in rat 1 week after unilateral injection of ibotenic acid-induced lesion (C–D), respectively, in the unlesioned (contra) and lesioned (ipsi) side of hippocampal formation. Note the increased levels of CaSR mRNA expression in the hippocampal CA1–CA3 pyramidal cell layers and dentate gyrus (DG) of kainate treated rat (B) and in the contralateral hippocampus (contra) of ibotenic injected rats. Autoradiophotograms in dark field (E, I) and in bright field (F, J) showing CaSR mRNA expression in the area of lesion 1 week after unilateral intrahippocampal ibotenic acid injection (E–F) or in the area of needle insertion-induced mechanical lesion (G–H), and (I, J) brain section hybridized with sense probe respectively in the hippocampus of ibotenic lesion and in the cerebral cortex of needle lesion. The arrows indicate cells positive for CaSR mRNA expression. Scale bar: (A–D) 300 μm; (E, I) 200 μm; (F, J) 50 μm.

A 628-bp fragment, encompassing nucleotides 3168-3795 (GenBank accession number NM_016996) for CaSR, DNA fragments were separated by electrophoresis, recovered from agarose gel with the GeneClean II Kit (Bio 101, CA), subcloned in pCR-Script SK (+) (Stratagene La Jolla, CA, USA). The recombinant plasmids were then linearized with *Eco*R1

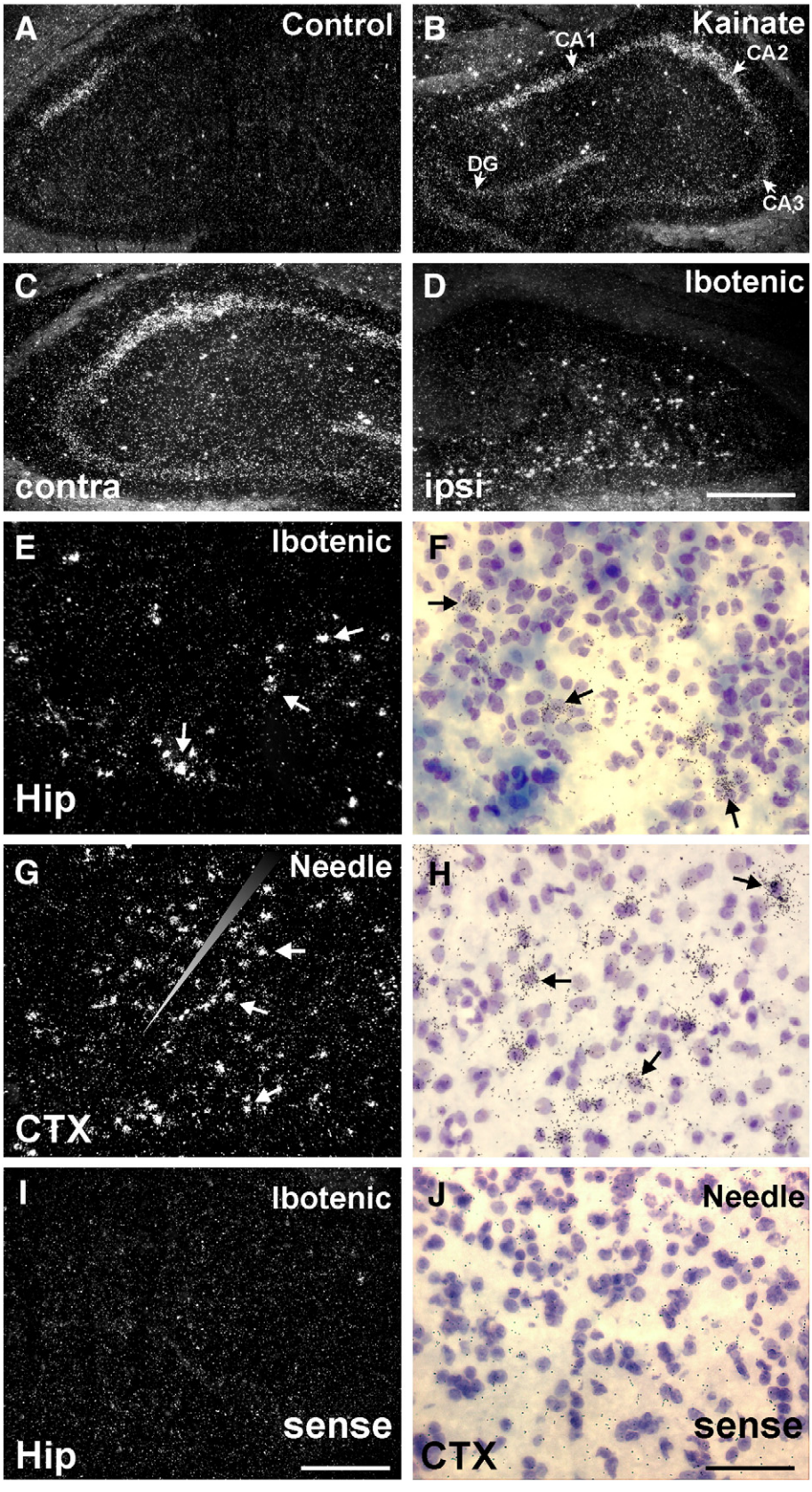

Table 2 – Relative CaSR mRNA levels per cell, and percentage of cells expressing CaSR mRNA in the cerebral cortex and hippocampal formation of rat brain 1 week post-injury.

Brain regions	% of CaSR mRNA labeled cells				Grains density per cell			
	Control	Ibotenic Contra [a]	Kainate	Needle	Control	Ibotenic Contra [a]	Kainate	Needle
Cerebral Cortex	6.8±2.5	–	–	14.2±3.4 $P<0.01$	124.8± 18			55.8±7.3 $P<0.00001$
CA1 pyramidal layer	15±3.8	47.2±10.3 $P<0.001$	45±9.7 $P<0.001$	–	5±2.5	12.2±3.9 $P<0.01$	9±2.6 $P<0.05$	_
CA2 pyramidal layer	51.6±5.7	64.4±7.8 $P<0.002$	60±2.9 $P<0.002$	–	50±4.1	75±9.3 $P<0.001$	72.2±7.2 $P<0.001$	_
CA3 pyramidal layer	17.4±3.6	32.6±7.5 $P<0.01$	31.8±4.6 $P<0.001$	–	4.2±1.3	12±3.1 $P<0.001$	10.2±4.3 $P<0.02$	_
Dentate gyrus	0	25±4	20±2.4	–	0	10±1.8	7±1.2	–
Ibotenic lesioned area	12.7±3.3	35.8±7.4 $P<0.001$		–	38.2±4.4	51±8.3 $P<0.02$	–	–

The relative CaSR mRNA levels and the percentage of cells expressing CaSR mRNA have been evaluated as described in material and methods. [a] Ibotenic contra indicates the contralateral hippocampal formation to lesioned one. Statistical analysis of Means±SD. The data were evaluated by *t-test*.

and transcribed with T3 RNA polymerase for the antisense probes. To obtain the sense probes, the same plasmids were linearized with *Sac*1 and transcribed with T7 RNA polymerase.

For transcription template was incubated 60 min at 37 °C in the presence of a transcription buffer made of 40 mM Tris–HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine and supplemented with 12.5 nmol ATP, CTP and GTP, 500 pmol UTP and 125 pmol ^{35}S α-UTP (Perkin-Elmer Italia, cod. NEG039H), 1 IU/μl RNAse inhibitor and 1 IU/μl of specific polymerase. The cDNA template was digested by adding 20 ng/ml DNAse I at 37 °C for 30 min. Transcripts were purified using ProbeQuant columns (ProbeQuant™ G-50 Micro Columns, cod. 27533501, GE Healthcare Europe GmbH). Control of the hybridization specificity of the cRNA riboprobes was performed using sense ^{35}S-labeled riboprobes.

Serial brain coronal cryostat sections of 14 μm were thawed onto 3-aminopropyl ethoxysilane-coated slides for in situ hybridization with radiolabeled probes as follows. Following

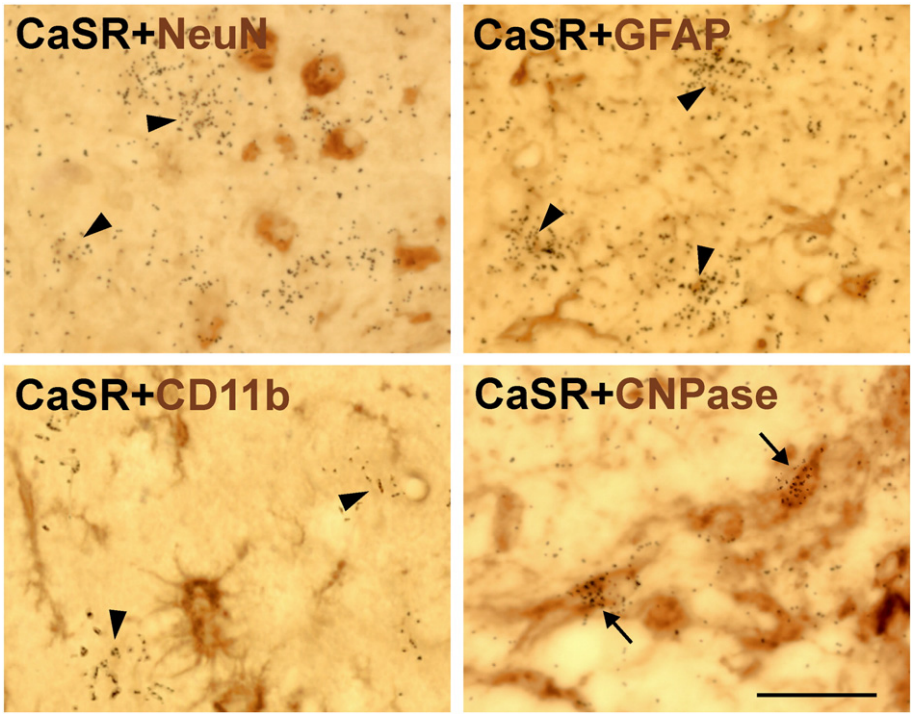

Fig. 8 – Analysis of cell type-expressing CaSR mRNA in the injured area 1 week after unilateral intrahippocampal ibotenic acid injection. Autoradiophotograms from coronal sections showing in high magnification that in the area of lesion CaSR mRNA colocalize with CNPase marker (arrows) and does not colocalize with NeuN, GFAP or CD11b markers (Arrowhead). Scale bar: 30 μm.

fixation in 4% paraformaldehyde for 15 min, slides were rinsed twice in PBS and once in distilled water. Tissue was deproteinated in 0.2 M HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M ethanolamine for 20 min and dehydrated with increasing concentrations of ethanol. Slides were incubated 16 h in a humidified chamber at 52 °C with 8×105 cpm of probe in 70 μl hybridization cocktail (50% formamide, 20 mM Tris–HCl (pH 7.6), 1 mM EDTA pH 8.0, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly-A-RNA, 1× Denhardt's solution and 10% dextran sulfate). Slides were washed twice in 1×SSC at 62 °C for 15 min and then in formamide and SSC (1:1) at 62 °C for 30 min. After an additional washing in 1×SSC at 62 °C, single-stranded RNA was digested by RNAse treatment (10 mg/ml) for 30 min at 37 °C in 0.5 M NaCl, 20 mM Tris–HCl pH 7.5, 2 mM EDTA. Tissue was washed twice with 1×SSC at 62 °C for 30 min before dehydration in ethanol and air-drying. For regional localization of mRNA, hybridized sections were exposed for 3 weeks to BioMax MR Film (Kodak, cod 891 2560) and subsequently coated with Emulsion Type NTB (Kodak, cod. 8895666) diluted 1:1 in water, stored in desiccated light-tight boxes at 4 °C for 4 weeks. Slides were developed with D19 (Kodak, cod. 1464593), fixed with Kodak Fixer (Kodak, cod. 1971746) and counterstained with cresyl violet, rinsed in PBS, dehydrated through graded alcohols, cleared in xylene, and cover-slipped in DPX mountant.

4.3. *Double labeling by combining in situ hybridization for CaSR mRNA with immunolabeling for neuronal and non-neuronal cell markers*

We have used a combination of in situ hybridization and immunohistochemical technique to identify the cellular type-expressing CaSR mRNA in the rat brain. The rats under deep anesthesia were perfused through the aorta with 0.9% saline followed by perfusion fixation with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid. Brains were removed, post-fixed in the same fixative for 2 h, and then transferred into 10% sucrose in 0.1 M phosphate buffer (PBS), overnight at 4 °C. Brain coronal cryostat sections of 20 μm thick were thawed onto 3-aminopropyl ethoxysilane- and gelatin-coated slides. Immunohistochemistry labeling was performed immediately after the last washing of the in situ hybridization procedure. Sections were washed with PBS and incubated for 15 min in blocking buffer consisting of 2.5% normal goat serum and 0.3% Triton X-100 in PBS. Subsequently, sections were incubated overnight at 4 °C in the presence of the primary antibody diluted in PBS supplemented with 1.5% blocking serum. The following mouse monoclonal antibodies (Chemicon, Temecula, CA, USA) and dilutions were used: (a) anti-neuron-specific DNA-binding protein (anti-NeuN, cod. MAB377) diluted 1:500; (b) anti-glial fibrillar acid protein (anti-GFAP, cod. MAB360) diluted 1:500, anti-2′3′-cyclic nucleotide 3′-phosphohydrolase (anti-CNPase, cod. MAB326); anti-rat integrin-aM (anti-CD11b, cod. MAB1405) diluted 1:200. Sections were then washed three times for 5 min in PBS and incubated at room temperature for 1 h with a biotinylated antimouse antiserum processed as per the Vectastain Universal Quick Kit (PK-8800), and developed with diaminobenzidine (DAB, #K3468; Dako). The reaction was stopped in Tris–HCl buffer and after a short washing with H_2O, the sections were dehydrated in an ascending alcohol series, coated in NTB emulsion and processed as described above for autoradiographic development.

4.4. *Quantitative evaluation of labeled cell*

The number of labeled cells was estimated by counts made by sampling in three representative sections the area of interest (identified according to the atlas of Paxinos and Watson, 1999). All cells inside of selected area were counted and therefore evaluated the percentage of labeled cells. All the counts were carried out in a double blind manner and by mean of computerized image analysis software (IAS-Counter, Delta Sistemi, Rome, Italy). Computerized image analysis was performed using software coupled to a Leica microscope equipped with digital video camera (Spot-RT Slider, Diagnostic Instruments, Mi, USA).

The labeling and the relative levels of mRNA expression (intensity) per cells were evaluated from emulsion dipped slides by evaluation of silver grains over the individual cells, using image analysis system (IAS-Counter, Delta-Sistemi, Roma, Italy). A correction factor for overlapping grains was included. For each brain area examined, the grains of cells in three different sections were counted. Cells were considered positive when the amount of silver grains after subtraction of background was exceeding 5 grains.

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