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Research highlights

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- Different GABAergic populations of the striatum expressed Adcy1 and Adcy5 mRNA in a differential manner.

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Neuroanatomical distribution and neurochemical characterization of cells expressing adenylyl cyclase isoforms in mouse and rat brain

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ABSTRACT

Transmembrane Adenylyl cyclases (Adcy) are involved in the regulation of multiple brain processes such as synaptic plasticity, learning and memory. They synthesize intracellular cyclic adenosine monophosphate (cAMP) following activation by G-protein coupled receptors. We examined the neuroanatomical distribution of the nine Adcy isoforms in rat and mouse brain by *in situ* hybridization, as well as their location in glutamatergic, GABAergic and cholinergic neurons in several mouse brain areas by double *in situ* hybridization. The Adcys are widely distributed throughout the brain in both rat and mouse, being especially abundant in cortex, hippocampus, thalamic nuclei, the olfactory system and the granular layer of the cerebellum. Double-labeling experiments showed that Adcy isoforms are differently expressed in glutamatergic, GABAergic and cholinergic neuronal cell populations. We report the neuroanatomical distribution of the nine known Adcy isoforms in rat and mouse brain and their cellular localization.

Research highlights

- Differences in mRNA expression of some Adcy isoforms (Adcy1, Adcy2, Adcy5 and Adcy8) were observed between rat and mouse hippocampal fields.
- Adcy2 mRNA expression was found expressed in all striatal cholinergic cells.
- Different GABAergic populations of the striatum expressed Adcy1 and Adcy5 mRNA in a differential manner.

1. INTRODUCTION

cAMP has a key role as an intracellular messenger, mediating the effects of extracellular signals in various tissues. In brain, cAMP is involved in sensory functions, synaptic plasticity, learning and memory. Intracellular levels of cAMP are controlled by its synthesis, catalyzed by the enzyme adenylyl cyclase, and by its degradation through the action of cyclic nucleotide phosphodiesterases (PDEs).

There are currently ten identified isoforms of the Adenylyl cyclase (Adcy) enzymes: transmembrane Adcys 1-9 regulated by a host of hormones, neurotransmitters and other regulatory molecules through interaction with G protein-coupled receptors, playing an important role in neurotransmission in the CNS (Adcy 1-9) and soluble Adcy regulated by bicarbonate. Increased levels of cAMP in different signaling pathways regulate neuronal mechanisms like synaptic plasticity, learning and memory, and neuroinflammatory responses. Transmembrane Adcy mRNAs are widely expressed throughout the brain, especially in olfactory bulb, hippocampus and cerebellum (Matsuoka et al., 1992; Visel et al., 2006) and present in other peripheral organs (Defer et al., 2000a; Risoe et al., 2007). The role of some Adcy isoforms in a number of brain processes, such as Adcy1 and Adcy8 in learning and memory (Mons et al., 1999; Conti et al., 2007), and Adcy5 in dopamine receptor signaling in the striatum (Lee et al., 2002) are broadly understood, but little is known about the function of other Adcys in the CNS. To date, nine Adcy isoforms have been cloned and identified in various tissues (Hanoune and Defer, 2001). Adcy isoforms are classified, according to their protein sequence homologies and function, into four groups (Patel et al., 2001; Cooper, 2005), designated as A, B, C and D (Visel et al., 2006). Group A consists of Adcy1, Adcy3 and Adcy8, which are stimulated by Ca^{+2}/CaM (Cali et al., 1994; Krupinski and Cali, 1998). Adcy1 and Adcy8 are expressed in neuronal tissue (Wong et al., 1999), while Adcy3 is mostly expressed in the olfactory system (Bishop et al., 2007). Adcy2, Adcy4 and Adcy7, forming group B, are Ca^{+2}-insensitive but are stimulated by the $G_{\beta\gamma}$ subunit (Feinstein et al., 1991). Adcy4 and Adcy7 are widely distributed

in several tissues (Mons et al., 1998), while Adcy2 is mainly found in lung and brain. Group C contains Adcy5 and Adcy6, expressed mostly in heart and brain, and inhibited by Ca^{+2} and the $G_{i\alpha}$ subunit (Defer et al., 2000). Adcy9, expressed mainly in brain, belongs to group D and is the only isoform that is not activated by forskolin, although it is responsive to calcineurin (Premont et al., 1996). The uneven tissue distribution of the individual Adcy isoforms and the diversity of their regulatory features may reflect a specific function of this effector molecule in determining the routing of signals to the cAMP pathway.

Knowledge of the cell location of the Adcys in brain is an important step towards understanding their function. Here, we analyze the regional expression of mRNA coding for the different Adcy isoforms in adult rat and mouse brain. By in situ hybridization, we determined in different rat and mouse brain areas the location of mRNA coding for nine different Adcys and the type of neuron in which they are expressed. Our study revealed that each Adcy mRNA has a distinct distribution, with differences between species.

Key words: *in situ* hybridization, cAMP, adenylyl cyclase, cholinergic cells, glutamatergic cells, GABAergic cells

2. EXPERIMENTAL PROCEDURES

2.1. Tissue preparation

Male Wistar rats (n=5) (200-300g) were purchased from Iffa Credo (Lyon, France) and adult male C57BL6 mice (n=5) (15-20g) from Charles River Laboratories (Wilmington, MA, USA). Animal care followed the Spanish legislation on “Protection of animals used in experimental and other scientific purposes”, which is in line with the European (E.E.C) regulations (O.J. of E.C. L358/1 18/12/1986). Rats were killed by decapitation and mice by cervical dislocation. The brains were rapidly removed, frozen on dry ice and kept at -20ºC. Tissue sections, 14 μm thick, were cut on a microtome-cryostat (Microm HM550 OM, Walldorf, Germany), thaw-mounted on APTS (3-

aminopropyltriethoxysilane; Sigma, St Louis, MO, USA)-coated slides, and kept at -20ºC until used.

2.2. Hybridization probes

The 45 base-oligodeoxyribonucleotides used for the detection of Adcy mRNAs are listed in Table 1. These regions were chosen because they share no similarity with the other Adcy isoforms.

Glutamatergic cells were recognized by the presence of the mRNA coding for both vesicular glutamate transporters (vGluT1 and vGluT2): vGluT1 with two oligonucleotides complementary to bases 127–172 and 1756–1800 (GenBank acc. no U07609) and vGluT2 with two oligonucleotides complementary to bases 466–510 and 2156–2200 (GenBank acc. no AF271235). GABAergic cells were identified by the presence of the enzyme synthesizing GABA, glutamic acid decarboxylase (GAD), which is found in adult brain as two major isoforms: GAD65 and GAD67. Two oligonucleotides for each isoform mRNA were made: bp 159–213 and 514–558 (GenBank acc. no NM_012563) and bp 191–235 and 1600–1653 (GenBank acc. no NM_017007). Cholinergic cells were distinguished by the presence of choline acetyltransferase (ChAT) mRNA with two oligonucleotides complementary to bases 571–618 and 1321–1368 of the rat ChAT cDNA sequence (Ishii et al., 1990).

The oligonucleotides were all synthesized and HPLC purified by Isogen Bioscience BV (Maarsen, The Netherlands). Evaluation of the oligonucleotide sequences with the basic local alignment search tool (BLAST) of EMBL and GenBank databases indicated that the probes show no significant similarity with mRNAs other than their corresponding targets in rat and mouse.

Oligonucleotides for Adcy mRNAs were labeled at their 3'-end by [α-^{33}P]dATP (3000 Ci/mmol, New England Nuclear, Boston, MA, USA) for the *in situ* hybridization histochemistry experiments and terminal deoxynucleotidyltransferase (TdT, Oncogene Research Products, San Diego, CA, USA), purified with the QIAquick Nucleotide Removal Kit (QIAGEN GmbH, Hilden, Germany) (Tomiyama et al., 1997). For the colocalization studies vGluT, GAD and ChAT oligonucleotides were labeled with Dig-11-dUTP and TdT (Roche Diagnostics, Manheim, Germany) according to a previously described procedure (Schmitz et al 1991).

2.3. In situ hybridization histochemistry procedure

The protocols for single- and double-label *in situ* hybridization histochemistry were based on previously described procedures (Tomiyama et al., 1997; (Landry et al., 2000) and have been already published (Serrats et al., 2003). Frozen tissue sections were brought to room temperature, fixed for 20 min at 4ºC in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1X PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3X PBS at room temperature, twice for 5 min each in 1X PBS, and incubated for 2 min at 21 °C in a solution of predigested pronase (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/ml in 50 mM Tris–HCl pH 7.5, 5 mM EDTA.

Enzyme activity was stopped by immersion for 30 s in 2 mg/ml glycine in 1X PBS. Tissues were finally rinsed in 1X PBS and dehydrated through a graded series of ethanol. For hybridization, radioactively and non-radioactively labeled probes were diluted in a solution containing 50% formamide, 4X SSC (1XSSC: 150 mM NaCl, 15 mM sodium citrate), 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 µg/ml yeast tRNA and 500 µg/ml salmon sperm DNA. The final concentrations of radioactive and digoxigenin-labeled probes in the hybridization buffer were in the same range (approximately 1.5 nM). Tissue sections were covered with hybridization solution containing the labeled probe/s, overlaid with Nescofilm coverslips (Bando Chemical Inc., Kobe, Japan) and incubated overnight at 42 °C in humid boxes. Sections were washed four times (15 min each) in 0.6 M NaCl, 10 mM Tris–HCl pH 7.5 at 60 °C, and once in the same buffer at room temperature for 30 min.

2.4. Development of radioactive and non-radioactive hybridization signals

Hybridized sections were treated as described by (Landry et al., 2000). Briefly, after washing, the slides were immersed for 30 min in a buffer containing 0.1 M Tris–HCl pH 7.5, 1 M NaCl, 2 mM MgCl2 and 0.5% bovine serum albumin (Sigma, Steinheim, Germany) and incubated overnight at 4°C in the same solution with alkaline-phosphatase-conjugated anti-digoxigenin-F(ab) fragments

(1:5000; Roche Diagnostics GmbH). Then, they were washed three times (10 min each) in the same buffer (without antibody) and washed twice in an alkaline buffer containing 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, and 5 mM $MgCl_2$. Alkaline phosphatase activity was developed by incubating the sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Diagnostics GmbH) diluted in 10 ml of alkaline buffer. The enzyme reaction was blocked by extensive rinsing in the alkaline buffer containing 1 mM EDTA. The sections were then briefly dipped in 70% and 100% ethanol, air-dried and dipped in Ilford K5 nuclear emulsion (Ilford, Mobberly, Cheshire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4º C for 6 weeks, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min and fixed in Ilford Hypam fixer (Ilford). For film autoradiography, some hybridized sections were exposed to Biomax-MR (Kodak) films for 2–4 weeks at -70°C with intensifying screens. Consecutive sections were stained with Cresyl Violet for anatomical reference.

2.5. Analysis of the results

Tissue sections were examined and cells quantified with an Olympus BX51 Stereo Microscope (Olympus, Tokyo, Japan) equipped with bright- and dark-field condensers for transmitted light. Cells were counted manually through the microscope with the help of Visiopharm Integrator System (Visiopharm Software, Hørsholm, Denmark). In this software a meander sampling tool was used to pass by the same counting areas twice, to be able to first count the amount of DIG-positive labeled cells in a predetermined area and then distinguish which of the cells also show silver grain accumulation.

Glutamatergic, GABAergic and cholinergic neurons were identified as cellular profiles exhibiting a dark precipitate (alkaline phosphatase reaction product) surrounding or covering the nucleus. The Adcy hybridization signal was considered positive when accumulation of silver grains over the stained cellular profiles was greater than three times that of the background.

2.6. Preparation on figures

Hybridized tissue section images from film autoradiograms were digitalized by a Wild 420 macroscope (Leica Microsystems, Wetzlar, Germany) equipped with a

digital camera (DXM1200 F, Nikon) and ACT-1 Nikon software. Microphotography was performed with an Olympus BX51 Stereo Microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DXM1200 F, Nikon). Figures were prepared for publication with Adobe Photoshop software (Adobe Software, San Jose, CA, USA). Contrast and brightness of images were the only variables we adjusted digitally. For anatomical reference, sections close to those used were stained with cresyl violet.

3. RESULTS

3.1. Controls for specificity of the probes

The specificity of the autoradiographic signal obtained in the *in situ* hybridization histochemistry experiments was confirmed by a series of routine controls (Pompeiano et al., 1992). For each mRNA under study, at least two different oligonucleotide probes complementary to different regions of the same mRNA were used independently as hybridization probes in consecutive sections of the same animal showing identical patterns of hybridization (Fig.1G, H). For a given oligonucleotide probe, addition in the hybridization solution of an excess of the same unlabeled oligonucleotide resulted in the complete abolition of the specific hybridization signal. The remaining autoradiographic signal was considered background (Fig.1D, E, and F). If the unlabeled oligonucleotide included in the hybridization was a different oligonucleotide, then the hybridization signal was not affected. The thermal stability of the hybrids was examined by washing at increasing temperatures: a sharp decrease in the hybridization signal was observed at a temperature consistent with the T_m of the hybrids (Fig.1A, B, and C).

In situ hybridization histochemistry provides reliable information concerning the relative abundance of a given mRNA species in different regions. However, caution must be taken in comparing the relative hybridization signals produced by different probes that detect different mRNAs. In addition to the actual abundance of the different mRNAs, the intensity of the hybridization signals observed, which is the parameter used in the present series of experiments for comparison between mRNAs, can be affected by other factors, such as

differences in the hybridization efficiency of the various probes or differences in the specific activities of the labeled probes.

3.2. Adcy mRNA distribution in rat and mouse brain

The distribution by *in situ* hybridization histochemistry of the different Adcy mRNA transcripts at coronal levels of the rat and mouse brain, illustrated in Figs. 2 and 3 and Tables 2 and 3, showed a selective expression pattern in certain cell layers and nuclei of the brain. Labeled brain nuclei were identified by comparison of the film autoradiograms with cresyl violet staining of the hybridized tissues. Adcy4 mRNA (Fig 2 and 3) could not be detected in any area of mouse or rat brain and will not be described below.

In situ hybridization histochemistry provides reliable information concerning the relative abundance of a given mRNA in different regions. However, caution must be taken when comparing the relative hybridization signals produced by different probes that detect different mRNA species. In addition to the actual abundance of the different mRNAs, the intensity of the hybridization signals observed can be affected by other factors, such as differences in the hybridization efficiency of the various probes or differences in the specific activities of the probes. Therefore, the results given here have to be interpreted with this caveat in mind when the abundance of the mRNA coding for the different Adcy enzymes is being compared.

3.2.1. Cortex

The expression pattern for all Adcy mRNAs was similar in both species.

A clearly different expression pattern in layers of the cortex was observed for transcripts of Adcy1, Adcy2, Adcy3, Adcy8 and Adcy9 in both rat (Fig. 2) and mouse (Fig. 3) brain. We also detected a homogeneous hybridization signal in all cortical cell layers for Adcy5 and Adcy6 and very low for Adcy7 mRNAs.

3.2.2. Olfactory system

Olfactory tubercle and piriform cortex were the two brain regions of the olfactory system that we examined in coronal sections of rodent brain in this work (see Tables 2 and 3). The mRNA levels of Adcy5 and Adcy9 isozymes were very high and moderate, respectively, in both rat and mouse (Figs. 2E1, 2I1, 3E1,

3I1). We also observed a very low hybridization signal for Adcy7 in the mouse brain sections. In the case of the piriform cortex, we detected the expression of transcripts of all Adcy genes (Figs. 2 and 3) except Adcy7 and Adcy3, which had a very low hybridization signal.

3.2.3. Striatum

The striatum nucleus is the main expression site of Adcy5 mRNA in both species (Figs. 2E1 and 3E1). We also found a strong and moderate hybridization signal in this brain nucleus for Adcy3 (Figs. 2C1 and 3C1) and Adcy9 (Figs. 2I1 and 3I1) transcripts, respectively. In rat brain, Adcy1 mRNA was expressed at moderate levels in the striatum (Fig. 2A1), whereas in mouse the transcript was present but at lower levels (Fig. 3A1). In the case of Adcy6 mRNA, the hybridization signal in striatum was moderate in coronal sections of mouse brain (Fig. 3F1), but we did not detect it in rat sections (Fig. 2F1).

3.2.4. Amygdala

We found moderate levels of mRNA expression in both species for all Adcy isozymes except Adcy9, which had a strong hybridization signal in rat amygdala (Fig. 2I2). Low or no expression of Adcy7 and Adcy8 mRNAs was found in this brain region.

3.2.5. Limbic areas

Moderate or strong expression for all Adcy mRNAs was detected in at least one of the three fields of the cornu ammonis (CA1, CA2, CA3) and the dentate gyrus (DG) of the hippocampus of both species (Figs. 2 and 3), except for Adcy7, which was not present in mouse.

A strong Adcy1 mRNA hybridization signal was present in CA2 and DG in both rat and mouse brain and in the CA1 field of mouse (Fig. 4A1 and 4A2). Adcy2 mRNA was found at high hybridization levels in CA2 and DG of both species. We also observed a moderate hybridization signal in the CA1 region of rat coronal sections (Fig. 4B1). We found high hybridization signals of Adcy3 in CA2 and CA3 and moderate ones in CA1 and DG, in both rat and mouse (Figs. 2C2 and 3C2). There were no differences in mRNA expression levels between the two species in this case. There was a moderate hybridization signal for

Adcy5 in the CA2 regions in both rat and mouse hippocampus. There was a low level of expression of Adcy5 mRNA in CA1, CA3 and DG of rat brain sections (Fig. 4C1), contrasting with its non-detection in the same hippocampal fields in mouse (Fig. 4C2). Adcy6 mRNA was expressed in all fields of rat and mouse hippocampus, though at a higher level in CA2 and DG (Figs. 2F2 and 3F2). There were no differences between the two species. Although we found expression of Adcy8 in all regions of the hippocampus, the CA1 field showed a remarkably high hybridization signal in mouse (Fig. 4D2). All three fields of CA and DG had a very high hybridization signal for Adcy9 mRNA in both rat and mouse brain sections (Figs. 2I2 and 3I2).

3.2.6. Thalamus and Hypothalamus

The mRNAs coding for Adcy2, Adcy3, Adcy5 and Adcy6 are weakly and uniformly expressed through the thalamus in both rat and mouse, whereas Adcy1, Adcy7, Adcy8 and Adcy9 mRNAs are present in both at high levels. Adcy1 mRNA expression in some thalamic nuclei was different between rat and mouse. We observed a strong hybridization signal for Adcy1 in the mouse posterior thalamic nuclear group (Fig. 5B), which was weaker in rat (Fig. 5A). The signal in the ventrolateral geniculate nucleus was also stronger in mouse brain. Adcy7 mRNA expression was only observed in mediodorsal thalamic nucleus of rat brain (Fig. 2G2). The moderate Adcy2 hybridization signal in the thalamic region was uniformly distributed in both species (Figs. 2C2 and 3C2), being higher in rat. In the hypothalamic nuclei, we found moderate hybridization signals in Adcy5 and low ones in Adcy3, Adcy6, Adcy7, Adcy8 and Adcy9, in both mouse and rat (Figs. 2 and 3). There were, however, some differences between species in Adcy1 mRNA expression in the hypothalamus. There was no hybridization signal for Adcy1 in hypothalamic nuclei of mouse brain (Fig. 5B), whereas the expression appeared at low levels in rat brain (Fig. 5A). Very low levels of Adcy2 mRNA were seen in the hypothalamus of both species.

3.3. Adcy mRNA expression in glutamatergic cells

Glutamatergic cells were identified by the presence of the vesicular glutamate transporters vGluT1 and vGluT2 mRNAs and were detected simultaneously in

tissue sections by Dig-labeled oligonucleotide probes. The presence of Adcy mRNAs was determined by ^{33}P-labeled oligonucleotides.

A high proportion of the glutamatergic cells in the dentate gyrus and in pyramidal cell layers of the hippocampus (Fig. 6) were positive. We observed high levels of coexpression in limbic areas (CA1-CA3, DG) for Adcy1 (Fig. 6 A-C), Adcy2 (Fig. 6 D-F) and Adcy9 (Fig. 6 J-L). Results are summarized in Table 4. We detected around 70-80% of glutamatergic cells expressing these three Adcy types in the CA1 region of the hippocampus. Adcy8 was found in 40% of glutamatergic cells in the CA1 field of the hippocampus (Fig. 6G). In DG, a vast majority of the glutamatergic cells (80-100%) (Fig. 6C and 6F) expressed Adcy1 and Adcy2, whereas in the CA3 field around 30% of these glutamatergic cells expressed Adcy1 or Adcy2 mRNA (Fig. 6B and 6E). For Adcy9, only 30% of DG vGluT-labeled cells also expressed the mRNA for this isoform (Fig. 6L), whereas 80% of co-localization was detected in the CA1, CA2 and CA3 fields (Fig. 6J and 6K). Low or very low mRNA co-expression of the other Adcys studied was detected in the glutamatergic cell population of the limbic areas studied.

In the thalamic nuclei analyzed in this study (DGL and VPM), we found a high amount of Adcy1 mRNA in around 80% of the glutamatergic cells (Fig. 7A). A moderate percentage of vGluT-positive cells (50-60%) in this brain area co-expressed Adcy8 mRNA (Fig. 7B). Low or no co-localization was seen for the other Adcys.

In the piriform and cingulate cortex, most labeled cells (85%) were double-positive for vGluT and Adcy1 (Fig. 8A). The same proportion of glutamatergic cells (52 and 47%) expressed Adcy2 and Adcy8 mRNAs (Fig. 8B and 8C). A lower percentage of vGluT-positive cells (24-36%) also hybridized for Adcy3, Adcy5 and Adcy9 mRNAs (Fig. 8D).

3.4. Adcy mRNA expression in GABAergic cells

GABAergic cells were identified by the presence of glutamic acid decarboxylase (GAD65 and GAD67) mRNAs and were detected in tissue sections by using Dig-labeled probes. The presence of Adcy mRNAs was determined by ^{33}P-labeled oligonucleotides.

Most GABAergic cells in the dentate gyrus (Fig. 9C) and in the pyramidal cell layer of the hippocampus (Fig. 9A and 9B) also expressed Adcy1 mRNA. No or low co-expression was observed for other Adcy mRNAs in the hippocampus. We also found moderate co-localization, around 70%, for the Adcy1 isoform in the GABAergic cell population of piriform and cingulate cortex (Fig. 9D). Around 30-37% of co-localization was observed in these cortical areas for Adcy2 and Adcy8 mRNAs.

We detected two differentiated GABAergic cell populations in the striatum: high GAD mRNA-expressing cells (interneurons or large striatonigral projection neurons) and low GAD mRNA-expressing cells (medium-spiny projection neurons). Adcy5 was the only isoform expressed in about 70% of the low GAD mRNA-expressing cells (Fig. 10B). For high GAD mRNA-expressing neurons, only Adcy1 mRNA was highly expressed (in 80%) in the striatum (Fig. 10A). The results are summarized in Table 4.

3.5. Adcy mRNA expression in cholinergic cells

Cholinergic cells were distinguished by the presence of choline acetyltransferase (ChAT) mRNA. We detected these cells in the striatum by use of non-radioactive probes. The presence of Adcy mRNAs was determined by ^{33}P-labeled oligonucleotides.

All ChAT mRNA-positive cells in the striatum expressed Adcy2 mRNA (Fig. 11B): this was the only cell population in striatum where this enzyme subunit was expressed. Adcy1, Adcy5 and Adcy6 mRNAs were also detected in about 60% of the cholinergic cells of the striatum (Fig. 11 A, C and D). The results are summarized in Table 4.

4. DISCUSSION

In this work we have carried out a comparative analysis of the mRNA distribution of the nine Adcy isoforms in rat and mouse brain. This study constitutes, to our knowledge, the first detailed comparative study of these enzymes in rat and mouse brain.

We have also examined the presence of the mRNAs coding for these Adcy isoforms in glutamatergic, GABAergic and cholinergic cells in selected mouse

brain structures. Although it is known that some Adcy isoforms are involved in neural processes (Mons et al., 1998; Lee et al., 2002), here we discuss the possible role of other Adcys during learning and memory or striatum function.

4.1. Adcys mRNA expression in rodent brain

We found Adcy1 mRNA expressed mostly in the hippocampus, the cerebellum, thalamic nuclei and neocortical structures in mouse and rat brain, in agreement with previous studies (Xia et al., 1991a; Mons et al., 1998; Visel et al., 2006; Conti et al., 2007). Our results confirmed that Adcy2 mRNA is mostly expressed in limbic areas in agreement with (Mons et al., 1998; Baker et al., 1999; Visel et al., 2006), where it has an important role on spatial memory acquisition (Mons et al., 2003). Adcy3 mRNA is expressed widespread in the brain (Visel et al., 2006), but has important functions in the olfactory system, where is a key component of the odorant receptor signaling cascade (Zou et al., 2007). We do not found Adcy4 mRNA expression in rodent brain, confirming previous reports (Visel et al., 2006) where they used a labeled riboprobe. However, some authors found Adcy4 protein expression in mouse hippocampus, where it may play a crucial role in certain forms of synaptic plasticity (Baker et al., 1999). Adcy5 mRNA expression was high in the olfactory tubercle and the striatum, where it plays a key role in the dopaminergic signaling cascade (Mons et al., 1998; Lee et al., 2002; Visel et al., 2006). Widespread and moderate expression was observed for Adcy6 mRNA in rodent brain (Visel et al., 2006), but a high mRNA expression could also be detected in the choroid plexus. While some authors found Adcy7 protein expression in several brain nuclei (Mons et al., 1998), we could only detected Adcy7 mRNA expression in some thalamic and hypothalamic nuclei. The discrepancy of our results on Adcy7 with those found by Mons and coworkers (1998) in the rat brain expression pattern could be explained by the different methodology used. They detect the expression of Adcy7 by immunohistochemistry using polyclonal antibodies which could be rather different in terms of levels of expression or brain areas where they are localized (not only cell bodies can be labeled as in the *in situ* hybridization experiments) but also terminals and dendrites. Adcy8 mRNA was found abundantly expressed in the hippocampus, cerebellum, cortex, thalamus and hypothalamus, confirming previous reports (Cali et al., 1994; Conti et al., 2007).

Referring to Adcy9, its mRNA was abundantly observed in neocortex and hippocampus, as described previously (Premont et al., 1996; Antoni et al., 1998).

The distribution of the different nine Adcy isoform mRNAs in mouse brain was found to be fundamentally similar to that described previously in rat (Matsuoka et al., 1992; Matsuoka et al., 1997; Mons et al., 1998), in displaying predominant locations in brain areas involved in a variety of functions (motor, sensorial, etc.). Differences in the intensity of expression between some cell groups within both species were apparent and could be relevant to questions such as for example how rats and mice are used to model several aspects of the regulation of some processes through cAMP levels regulation such as those involved in memory or neuroinflammatory response. Among the regions displaying such differences were 1) the CA fields and dentate gyrus of the hippocampus for Adcy1, Adcy2, Adcy5 and Adcy8 and 2) the thalamus, where Adcy1 presented very high hybridization levels in some thalamic nuclei in mouse.

4.2. Hippocampus: learning and memory implications

The Ca^{+2}/calmodulin-stimulated Adcys, Adcy1 and Adcy8, have an essential role in synaptic plasticity and are required for learning and memory processes (Wong et al., 1999; Zhang et al., 2008; Masada et al., 2009). Double-knockout mice show no late phase-long term potentiation and are deficient in long-term memory (Wong et al., 1999). These two isoforms are expressed in areas associated with learning and memory (Xia et al., 1991; Conti et al., 2007), including neocortex and hippocampus.

The high co-localization observed for Adcy1 in glutamatergic and GABAergic neurons in the hippocampus points to the importance of this enzyme's role in the synaptic plasticity of glutamatergic and GABAergic hippocampal terminals. Since Adcy1 is neurospecific (Xia et al., 1991), it may be a useful drug target to modulate synaptic plasticity.

Other Adcy isoforms are also involved in learning and memory. Adcy2, a Ca^{+2}-insensitive/ $G_{\beta\gamma}$-stimulated Adcy, is found transcriptionally downregulated during early stages of spatial learning tasks (Mons et al., 2003). In addition, we found

this isoform highly expressed in glutamatergic CA1 neurons of control mice, which supports its possible role in learning tasks. Adcy9, which we found highly expressed in most CA1-CA2 and CA3 glutamatergic neurons, is a calcineurin-activated Adcy isoform. It is activated by Ca^{+2}, producing increased levels of cAMP in hippocampal neurons *in vitro* (Chan et al., 2005). For these, Adcy9 may contribute to synaptic plasticity, including some forms of learning and memory (Antoni et al., 1998).

4.3. Striatum: dopaminergic and cholinergic functions

We only found high mRNA expression of Adcy1 and Adcy5 in GABAergic cell population in this brain region. The striatum is the main expression site of Adcy5 mRNA, where it is required for D_2 dopamine receptor function (Lee et al., 2002). High co-expression of Adcy5, and dopamine D_1 and D_2 receptors, is found in this brain nucleus (Gortari and Mengod, 2009). D_1 receptors are coupled to G_s-stimulated Adcys, like Adcy1, or a non-Adcy effector system like phospholipase C (PLC); whereas D_2 receptors are coupled to $G_{i\alpha}$-inhibited Adcys, like Adcy5 (Sidhu and Niznik, 2000). Our results indicate that Adcy5 mRNA is mainly expressed in medium-spiny neurons (GABAergic interneurons) in the striatum. These neurons account for 90% of the GABAergic neurons in this brain region and express both D_1 and D_2 receptors. These results, in addition to previous reports (Gortari and Mengod, 2009), could indicate that Adcy5 is coupled with the D_2 dopamine receptor in GABAergic medium-spiny neurons of the striatum, and that Adcy5 in the striatum is involved in corticostriatal dopamine-dependent plasticity and striatum-dependent learning (Kheirbek et al., 2009). In the other GABAergic cell population found in the striatum, large striatonigral projecting neurons or fast-spiking neurons, we found that only Adcy1 mRNA was highly expressed. This may indicate that dopamine D_1 receptors, in this case, could be coupled to this enzyme.

Other types of neurons present in the striatum are the large spiny cholinergic interneurons, which account for 2% or less of the total neuronal striatal population. These cells, also called tonically active neurons, receive dopaminergic signals, have projecting axons to both populations of striatal GABAergic cells (Tepper and Bolam, 2004) and express D_1-like dopamine receptors. Adcy2 mRNA was found, surprisingly, highly expressed in all

cholinergic neurons of the striatum. This may represent the Adcy2-signaling pathway's involvement in cholinergic modulation of GABAergic neurons. The low densities of silver grains observed in around 50% of cholinergic interneurons for the other Adcy mRNAs like Adcy1, Adcy5 and Adcy6 suggests that Adcy2 is probably the most important Adcy present in these neurons, where it may be coupled with D_1-like dopamine receptors, but we cannot exclude the other Adcys and other receptors dependent on Adcy-cAMP signaling pathways. We discussed above that D_1-like receptors can also be coupled with Adcy1, and that Adcy5 or Adcy6 could be coupled with D_2 receptors, which are also found in cholinergic neurons in the striatum (Dawson et al., 1988; Alcantara et al., 2003). Furthermore, Adcys may also be involved in acetylcholine release from vesicles in these cells (Login, 1997).

5. CONCLUSIONS

The results reported here on the neuroanatomical and neuronal localization of the isoforms of Adcy mRNAs in rat and mouse brains is a starting point for a more profound study of these enzymes, which might lead to their consideration as new therapeutic targets. More co-localization studies between Adcys and G-protein-coupled receptors are needed in order to establish which Adcy isoform is coupled to which receptor in these neuronal pathways. Understanding the specific Adcy-effector system for each receptor could be useful for drug treatments with Adcy isoform-specific inhibitors (Pierre et al., 2009).

Figure Legends

Figure 1. Specificity controls of the hybridization signal obtained with two labeled oligonucleotides. Rat horizontal sections were hybridized with ^{33}P-labeled oligonucleotide rAC1/2 (**A-G**) or rAC1/3 (**H**). Thermal stability of the hybrids was examined by washing at 50ºC (**A**), 60ºC (**B**) and 80ºC (**C**). No hybridization signal remained after co-hybridizing each labeled oligonucleotide with an excess of the corresponding unlabeled oligonucleotide (rAC1/2): 100x (**D**), 500x (**E**) and 1000x (**F**). The same hybridization pattern is observed for the two oligonucleotide probes used in **G** and **H**.Scale bar = 400μm

Figure 2. Expression of Adenylyl Cyclase mRNA in rat brain. Macroscopic photographs of autoradiographic film images of coronal sections showing mRNA hybridization pattern of nine isoforms of adenylyl cyclases in Wistar rats. A1, A2 Adcy1 mRNA, B1, B2 Adcy2 mRNA, C1, C2 Adcy3 mRNA, D1, D2 Adcy4 mRNA, E1, E2 Adcy5 mRNA, F1, F2 Adcy6 mRNA, G1, G2 Adcy7 mRNA, H1, H2 Adcy 8 mRNA, I1, I2 Adcy9 mRNA. Amy, amygdala; CA, *Cornu Ammonis*; CP, choroid plexus; CPu, caudate-putamen; Cx, cortex; Hyp, hypothalamus; LG, lateral geniculate nucleus; MD, medial dorsal thalamic nucleus; Pir, piriform cortex; STh, subthalamic nucleus; Th, thalamus; Tu, olfactory tubercle; VPM, ventral posteromedial thalamic nucleus. Scale bar = 2mm.

Figure 3. Expression of Adenylyl Cyclase mRNA in mouse brain. Macroscopic photographs of autoradiographic film images of coronal sections showing mRNA hybridization pattern of nine isoforms of adenylyl cyclases in C57BL6 mice. A1, A2 Adcy1 mRNA, B1, B2 Adcy2 mRNA, C1, C2 Adcy3 mRNA, D1, D2 Adcy4 mRNA, E1, E2 Adcy5 mRNA, F1, F2 Adcy6 mRNA, G1, G2 Adcy7 mRNA, H1, H2 Adcy 8 mRNA, I1, I2 Adcy9 mRNA. Amy, amygdala; CA, *Cornu Ammonis*; cg, cingulum; CPu, caudate-putamen; Cx, cortex; DLG, dorsolateral geniculate nucleus; Hyp, hypothalamus; Pir, piriform cortex; Th, thalamus; Tu, olfactory tubercle; VLG, ventrolateral geniculate nucleus; ZI, zona incerta. Scale bar = 2mm.

Figure 4. Different expression of Adenylyl Cyclase mRNA in rat and mouse hippocampus. Macroscopic photographs of autoradiographic film images of coronal sections showing mRNA hybridization pattern of Adcy1 (**A**), Adcy2 (**B**), Adcy5 (**C**) and Adcy8 (**D**) in rat (**A1-D1**) and mouse (**A2-D2**) hippocampus. Note the differences in mRNA expression levels between species (black arrows). Scale bars = 300 μm and 500 μm.

Figure 5. Different expression of Adenylyl Cyclase 1 mRNA in rat and mouse in thalamic and hypothalamic nuclei. Macroscopic photographs of autoradiographic film images of coronal sections showing mRNA hybridization pattern of Adcy1 in rat (**A**) and mouse (**B**) thalamus and hypothalamus. Note the differences in mRNA expression levels between species (black and white arrows). Hyp, hypothalamus; Th, thalamus. Scale bars = 1mm.

Figure 6. Glutamatergic cells of limbic areas expressing adenylyl cyclase isoform mRNA in mouse brain. High-magnification photomicrographs showing the simultaneous detection of two species of mRNA by using digoxigenin-labeled probes for vGluT mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of Adcy isoforms Adcy1 (**A–C**), Adcy2 (**D–F**), Adcy8 (**G–I**) and Adcy9 (**J-L**) in the pyramidal layer of the hippocampus (CA1 field; **A, D, G, J**; CA3 field; **B, E, H, K**) and in the dentate gyrus of the hippocampus (**C, F, I, L**) of mouse brain. White arrowheads point to digoxigenin-labeled cells, black arrowheads to radioactively-labeled cells and double white and black arrowheads to double-labeled cells. CA, *Cornu Ammonis*; DG, dentate gyrus; vGluT, vesicular glutamate receptor. Scale bar = 100 μm.

Figure 7. Glutamatergic cells of thalamic nucleus expressing adenylyl cyclase isoform mRNA in mouse brain. High-magnification photomicrographs showing the simultaneous detection of two species of mRNA by using digoxigenin-labeled probes for vGluT mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of Adcy isoforms Adcy1 (**A**) and Adcy8 (**B**) in the ventral posterior thalamic nucleus of mouse brain. White arrowheads point to digoxigenin-labeled cells, black arrowheads to radioactively-labeled cells and

double white and black arrowheads to double-labeled cells. vGluT, vesicular glutamate receptor. Scale bar = 20 μm.

Figure 8. Glutamatergic cells of cingulate cortex expressing adenylyl cyclase isoform mRNA in mouse brain. High-magnification photomicrographs showing the simultaneous detection of two species of mRNA by using digoxigenin-labeled probes for vGluT mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of Adcy isoforms Adcy1 (**A**), Adcy2 (**B**), Adcy8 (**C**) and Adcy9 (**D**) in the cingulate cortex of mouse brain. White arrowheads point to digoxigenin-labeled cells, black arrowheads to radioactively-labeled cells and double white and black arrowheads to double-labeled cells. vGluT, vesicular glutamate receptor. Scale bar = 20 μm.

Figure 9. GABAergic cells of the hippocampus and cingulate cortex expressing adenylyl cyclase 1 isoform mRNA in mouse brain. High-magnification photomicrographs showing the simultaneous detection of two species of mRNA by digoxigenin-labeled probes for GAD65/67 mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of Adcy1 isoform in the pyramidal layer of the hippocampus (CA1 field; **A**; CA3 field; **B**), in the dentate gyrus of the hippocampus (**C**) and in the cingulate cortex (**D**) of mouse brain. Double white and black arrowheads point to double-labeled cells. CA, *Cornu Ammonis*; CgCx, cingulate cortex; DG, dentate gyrus; GAD, glutamic acid decarboxylase. Scale bar = 20 μm.

Figure 10. GABAergic cells of the striatum expressing adenylyl cyclase isoform mRNA in mouse brain. High-magnification photomicrographs showing the simultaneous detection of two species of mRNA by using digoxigenin-labeled probes for GAD65/67 mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of Adcy isoforms Adcy1 (**A**) and Adcy5 (**B**) in the striatum of mouse brain. White arrowheads point to low GAD65/67 mRNA-expressing cells, white arrows to high GAD65/67 mRNA-expressing cells, black arrowheads to radioactively-labeled cells and double white and black arrows and

arrowheads to double-labeled cells. GAD, glutamic acid decarboxylase. Scale bar = 20 μm.

Figure 11. Cholinergic cells of the striatum expressing adenylyl cyclase isoform mRNA in mouse brain. High-magnification photomicrographs showing the simultaneous detection of two species of mRNA by using ^{33}P-labeled oligonucleotide probes for Adcy1 (**A**), Adcy2 (**B**), Adcy5 (**C**) and Adcy6 (**D**) isoforms (silver grains) and digoxigenin-labeled probes for ChAT mRNA in the striatum of mouse brain. White arrowheads point to digoxigenin-labeled cells, black arrowheads to radioactively-labeled cells and double white and black arrowheads to double-labeled cells. ChAT, choline acetyltransferase. Scale bars = 100 μm and 20 μm in the magnification boxes.

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Table 1. List of oligonucleotides used.

mRNA	Oligonucleotide name	Accession number	Bp limits
AC1	rAC1/2	**XM_223616**	3555-3599
	rAC1/3	**XM_223616**	3612-3656
AC2	rAC2/1	**NM_031007**	2908-2952
	rAC2/2	**NM_031007**	2975-3019
	rAC2/3	**NM_031007**	2957-3001
AC3	rAC3/1	**NM_130779**	2751-2795
	rAC3/2	**NM_130779**	2533-2577
AC4	rAC4/1	**NM_019285**	2406-2450
	rAC4/2	**NM_019285**	3257-3301
	rAC4/3	**NM_019285**	2611-2655
	rAC4/4	**NM_019285**	2866-2910
	rAC4/5	**NM_019285**	112-156
	rAC4/6	**NM_019285**	1417-1461
AC5	rAC5/1	**NM_022600**	3061-3105
	rAC5/2	**NM_022600**	1340-1384
AC6	rAC6/2	**NM_012821**	656-700
	rAC6/3	**NM_012821**	601-645
	rAC6/4	**NM_012821**	500-544
AC7	rAC7/1	**XM_226333**	3760-3804
	rAC7/3	**XM_226333**	3809-3853
	rAC7/4	**XM_226333**	3710-3754
AC8	rAC8/1	**NM_017142**	3961-4005
	rAC8/2	**NM_017142**	4109-4153
AC9	rAC9/1	**XM_220178**	2786-2830
	rAC9/2	**XM_220178**	2945-2990

AC, adenylate cyclase.

Table(s)

Table 2. Expression of mRNAs encoding AC isoforms in different regions of rat brain.

Brain area	AC 1	AC 2	AC 3	AC 4	AC 5	AC 6	AC 7	AC 8	AC 9
Cerebral Cortex	++	++	+/++	-	+	+	+/-	+/-	++/+++
Olfactory system									
Olfactory tubercle	+/-	+/-	+	-	+++	+/-	-	-	++
Piriform cortex	+++	++/+++	+	-	++	++/+++	-	+++	+++
Basal ganglia and related areas									
Corpus callosum	-	+/++	-	-	+/-	+/-	-	+/-	+
Caudate-Putamen	+	+/-	++	-	+++	-	-	-	++
Amygdala	++	++	++	-	++	++	-	+	+/++
Limbic areas									
Ammon's horn									
CA1 (pyramidal cell layer)	+	+++	++	-	+	+	+	++	+++
CA2 (pyramidal cell layer)	+++	+	+++	-	++	+/++	+	+	+++
CA3 (pyramidal cell layer)	++	+	+++	-	+	+	+	+	+++
Dentate gyrus	+++	+++	++	-	++	++/+++	++	+	+++
Thalamus and Hypothalamus									
Med/Lat habenular nucleus	+	+/-	-	-	++	++	+++	++	++
Thalamic nucleus group	++	+/-	+	-	+	++	-	+/++	+/++
Reticular thalamic nucleus	++/+++	+	+	-	+	++	++	+	+
Medial hypothalamic nucleus	+	+	+	-	++	++	++/+++	+/-	+/-
Lateral geniculate nucleus	++/+++	+	+	-	++	++	++	+	+
Brainstem									
Superior colliculus	+	+	+	-	+	+	+	+	+
Inferior colliculus	+	+/-	+	-	+	+	+	+	+
Cerebellum	+++	++	++	-	+	+++	+/++	+++	++/+++
Circumventricular organs									
Choroid plexus	-	-	-	-	-	+++	+/-	++	-

Relative expression is indicated as follows: +++, very high; ++/+++, high; ++, moderate; +/++, low moderate; +, low; +/-, very low or hard to detect; -, not expressed. AC, adenylate cyclase.

Table 3. Expression of mRNAs encoding AC isoforms in different regions of mouse brain.

Brain area	AC 1	AC 2	AC 3	AC 4	AC 5	AC 6	AC 7	AC 8	AC 9
Cortex	++	++	++	-	+	++	+/-	++	++/+++
Olfactory system									
Olfactory tubercle	+	-	+/++	-	+++	++	+	+/-	++/+++
Piriform cortex	++	++/+++	++	-	+/++	+++	+/-	+++	+++
Basal ganglia and related areas									
Corpus callosum	-	+/-	+	-	-	+/++	+/-	++	+
Caudate-Putamen	+/-	+/-	++	-	+++	-	-	+/-	++
Amygdala	++	+	++	-	+	++/+++	+/-	+	+/++
Limbic areas									
Ammon's horn									
CA1 (pyramidal cell layer)	++/+++	+++	+++	-	+/-	++/+++	-	++/+++	+++
CA2 (pyramidal cell layer)	+++	++	+++	-	++	+++	-	++	+++
CA3 (pyramidal cell layer)	+/-	++	+++	-	+/-	++/+++	-	++/+++	+++
Dentate gyrus	+++	+++	++/+++	-	+/-	+++	-	++/+++	+++
Thalamus and Hypothalamus									
Med/Lat habenular nucleus	+/-	++	++	-	++	++	++	++/+++	+
Thalamic nucleus group	++	+/-	+	-	+	++	-	+++	++
Reticular thalamic nucleus	++/+++	++	+/++	-	+	++	++	+/++	+
Medial hypothalamic nucleus	-	+	++/+++	-	+/++	++/+++	+++	++/+++	+
Lateral geniculate nucleus	++/+++	+/-	+/++	-	++	++	++	+++	++
Brainstem									
Superior colliculus	+	+	+	-	+	+	+	+	+
Inferior colliculus	+	+	+	-	+	+	+	+	+
Cerebellum	+++	+/++	++/+++	-	+	+++	+	+++	++/+++
Circumventricular organs									
Choroid plexus	-	-	-	-	-	+++	-	-	-

Relative expression is indicated as follows: +++, very high; ++/+++, high; ++, moderate; +/++, low moderate; +, low; +/-, very low or hard to detect; -, not expressed. AC, adenylate cyclase.

Table(s)

Table 4. Quantification of the presence of Adenylate Cyclases mRNA in different neuronal populations in mouse brain.

		AC1	AC2	AC3	AC5	AC6	AC7	AC8	AC9
CPu	GAD high	71 ± 12	30 ± 31	11 ± 8	17 ± 3	13 ± 11	12 ± 10	10 ± 2	3 ± 3
	GAD low	13 ± 5	3 ± 0	6 ± 3	83 ± 6	4 ± 2	3 ± 1	4 ± 2	17 ± 1
	ChAT	66 ± 8	100 ± 0	34 ± 15	63 ± 4	58 ± 8	4 ± 1	2 ± 4	24 ± 6
Th	Glut	83 ± 6	0 ± 0	12 ± 2	23 ± 9	7 ± 4	10 ± 8	53 ± 19	28 ± 5
CA1	GAD	69 ± 9	44 ± 40	8 ± 7	7 ± 7	21 ± 19	20 ± 3	18 ± 11	18 ± 10
	Glut	88 ± 12	71 ± 8	13 ± 2	13 ± 8	7 ± 1	9 ± 8	44 ± 17	89 ± 1
CA2	GAD	79 ± 17	6 ± 10	6 ± 10	5 ± 8	0 ± 0	13 ± 0	0 ± 0	8 ± 14
	Glut	84 ±18	36 ± 29	10 ± 6	27 ± 13	9 ± 7	5 ± 3	37 ± 24	81 ± 3
CA3	GAD	64 ± 12	4 ± 4	11 ± 3	0 ± 0	11 ± 11	10 ± 14	0 ± 0	29 ± 12
	Glut	34 ± 14	38 ± 13	13 ± 3	23 ± 15	7 ± 1	7 ± 6	9 ± 6	80 ± 2
DG	GAD	82 ± 16	37 ± 17	11 ± 10	0 ± 0	11 ± 10	5 ± 7	10 ± 14	0 ± 0
	Glut	100 ± 0	84 ± 3	6 ± 9	18 ± 9	7 ± 2	9 ± 5	14 ± 4	34 ± 6
CgCx	GAD	74 ± 6	30 ± 9	9 ± 9	7 ± 2	18 ± 19	10 ± 9	37 ± 9	10 ± 5
	Glut	85 ± 1	52 ± 15	24 ± 17	21 ± 6	9 ± 5	9 ± 4	47 ± 16	36 ± 2

Quantification was performed in different brain regions of control mice brains. Data are the mean ± SD of four animals and represent the percentage of counted cells, GABAergic neurons (GAD), glutamatergic neurons (Glut) and cholinergic neurons (ChAT), expressing an AC isoform mRNA. The number of cells counted in each region was maintained for all sections analyzed. AC, adenylate cyclase; CA, *Cornu Ammonis*; CgCx, cingulate cortex; ChAT; choline acetiltransferase; CPu, caudate-putamen; DG, dentate gyrus; GAD, glutamic acid decarboxylase; Glut, glutamatergic; Th, thalamus.

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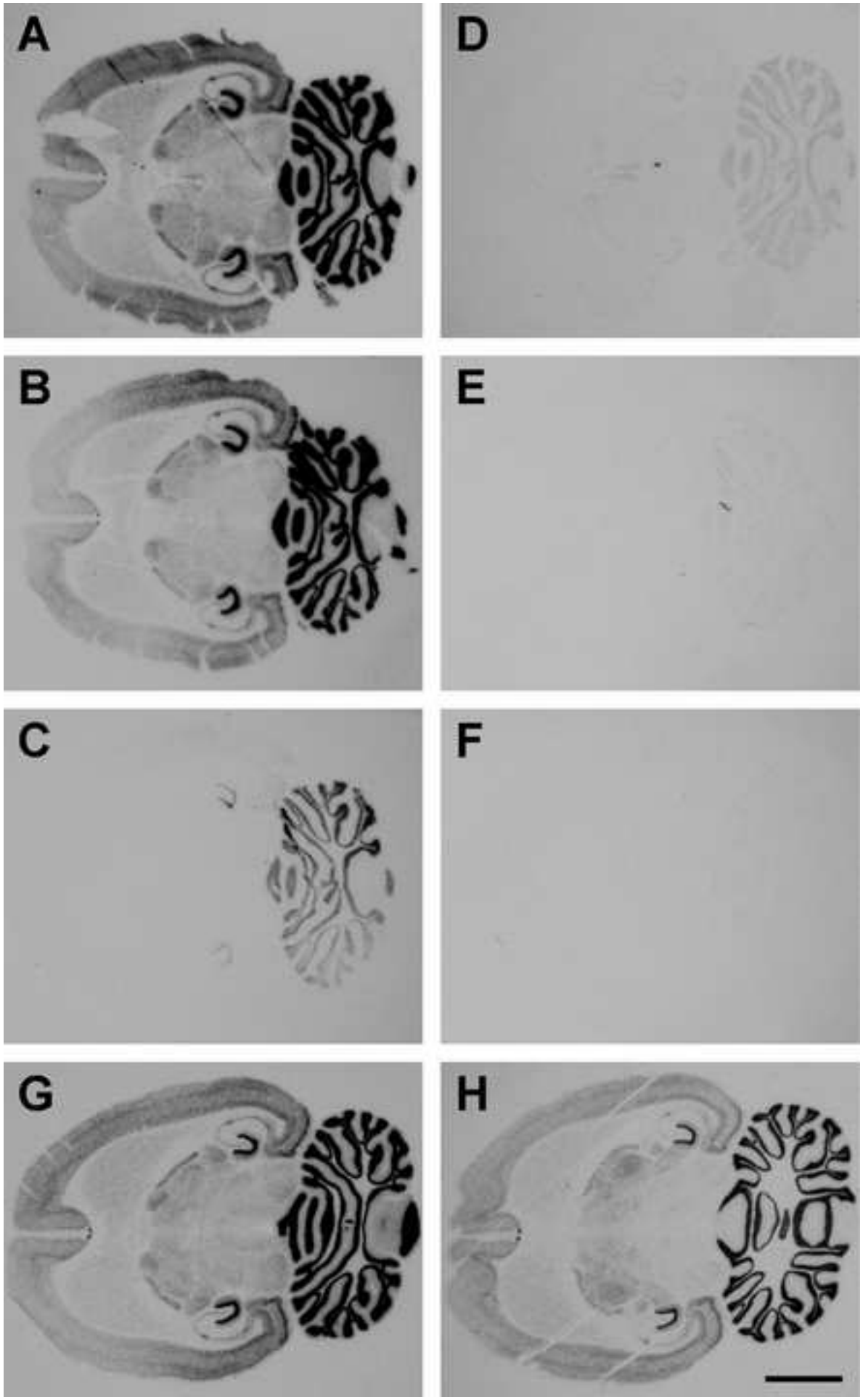

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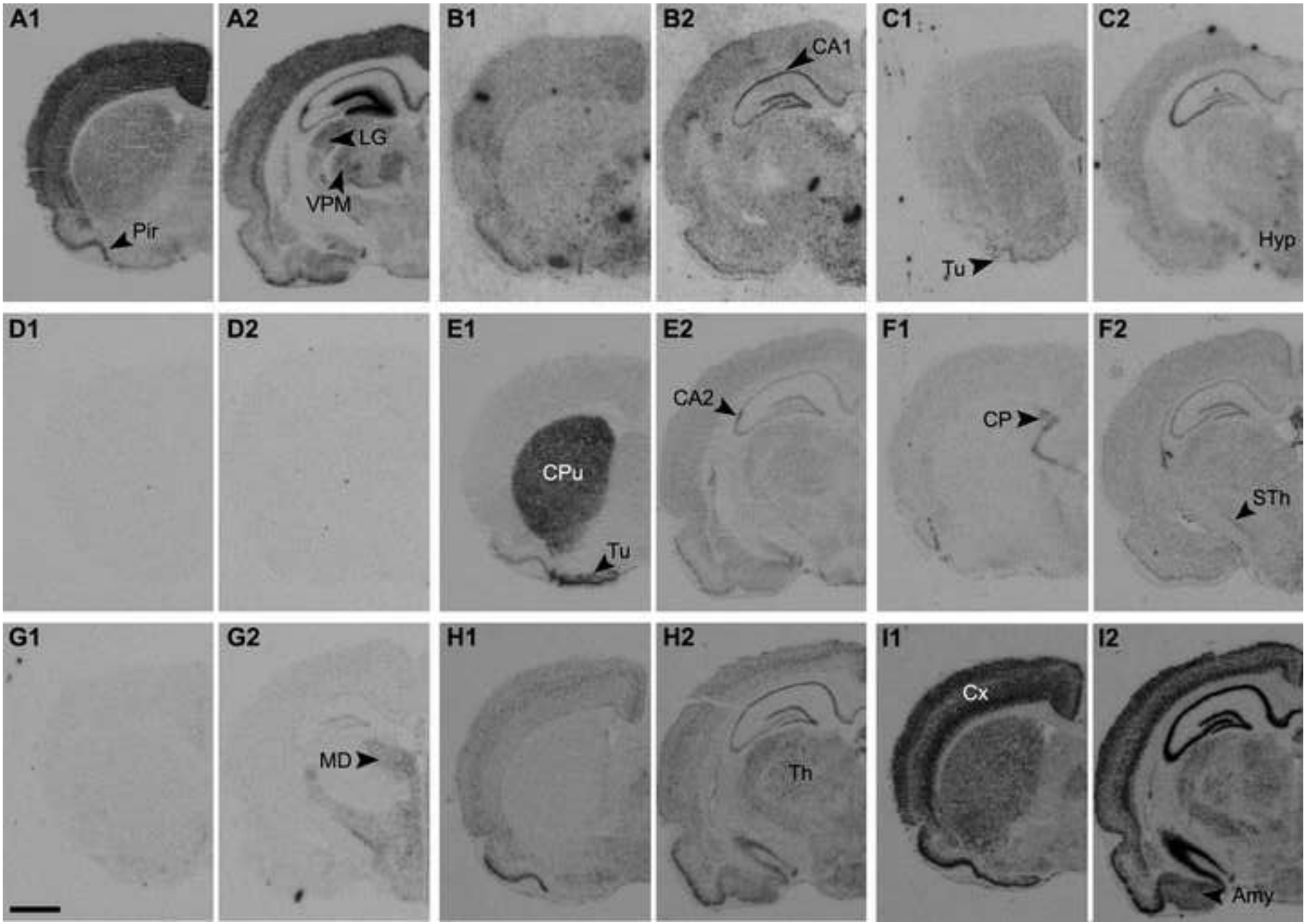

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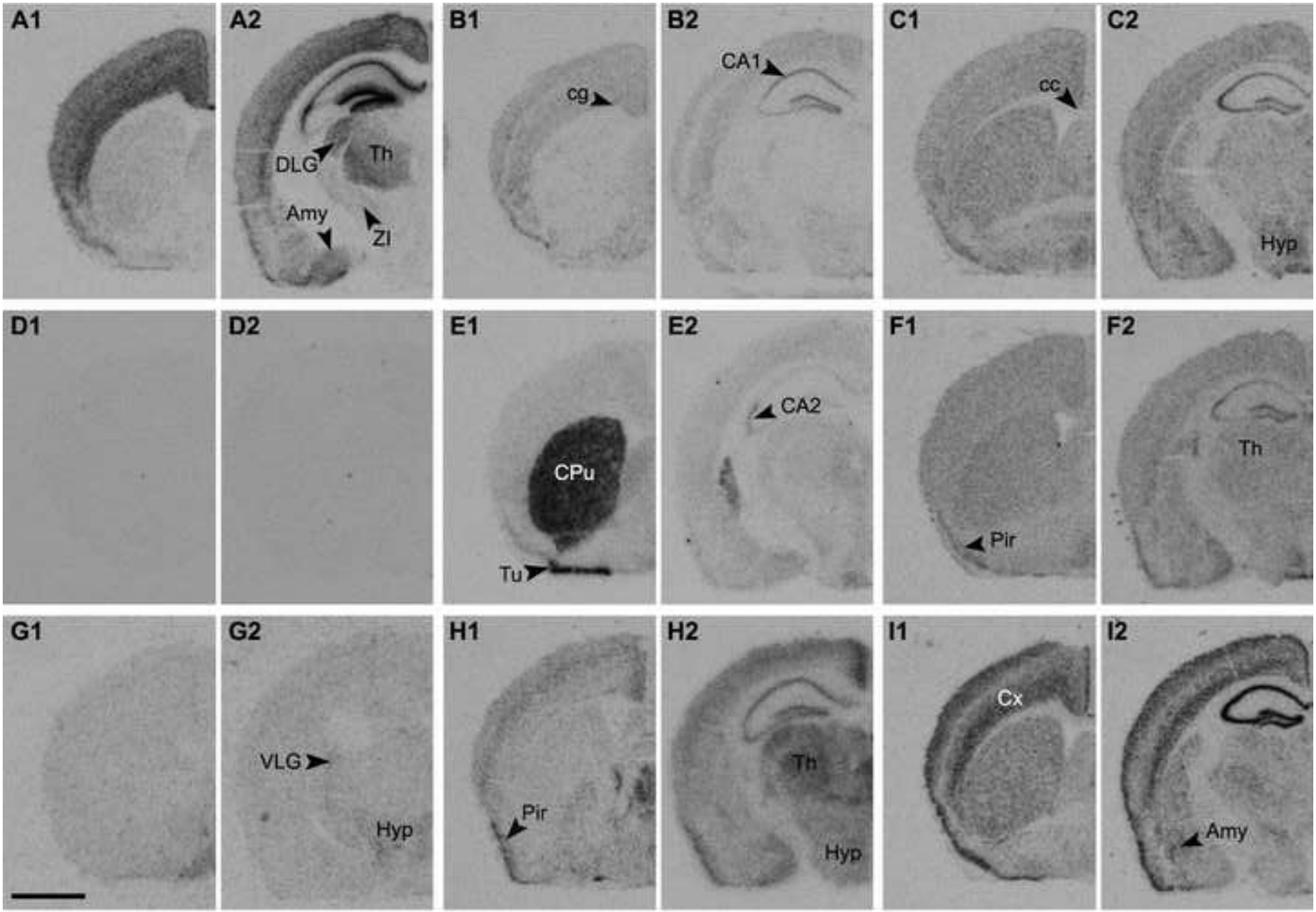

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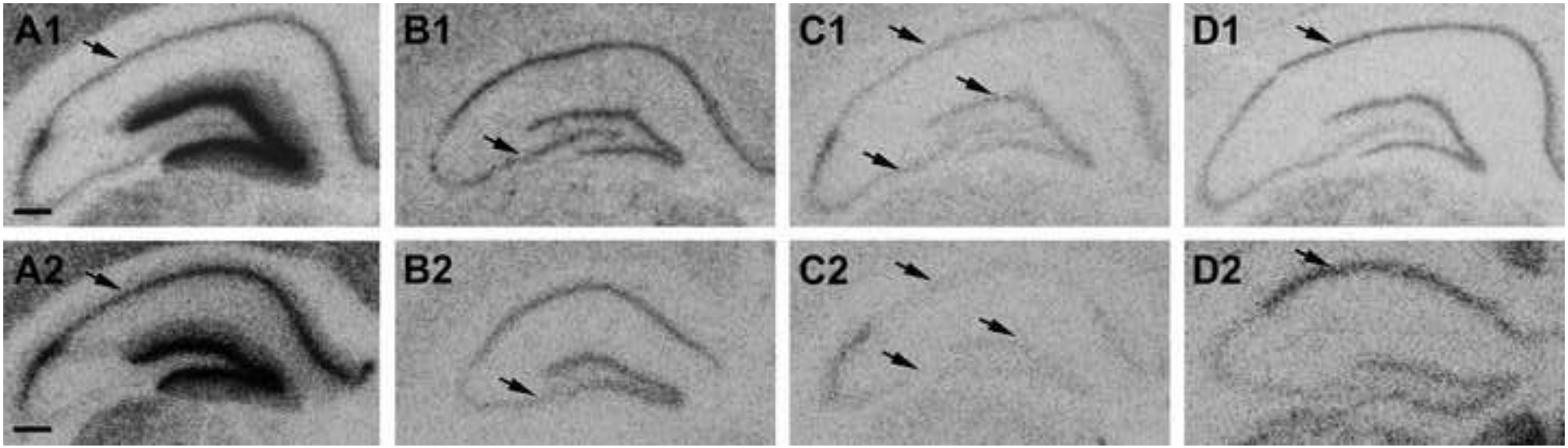

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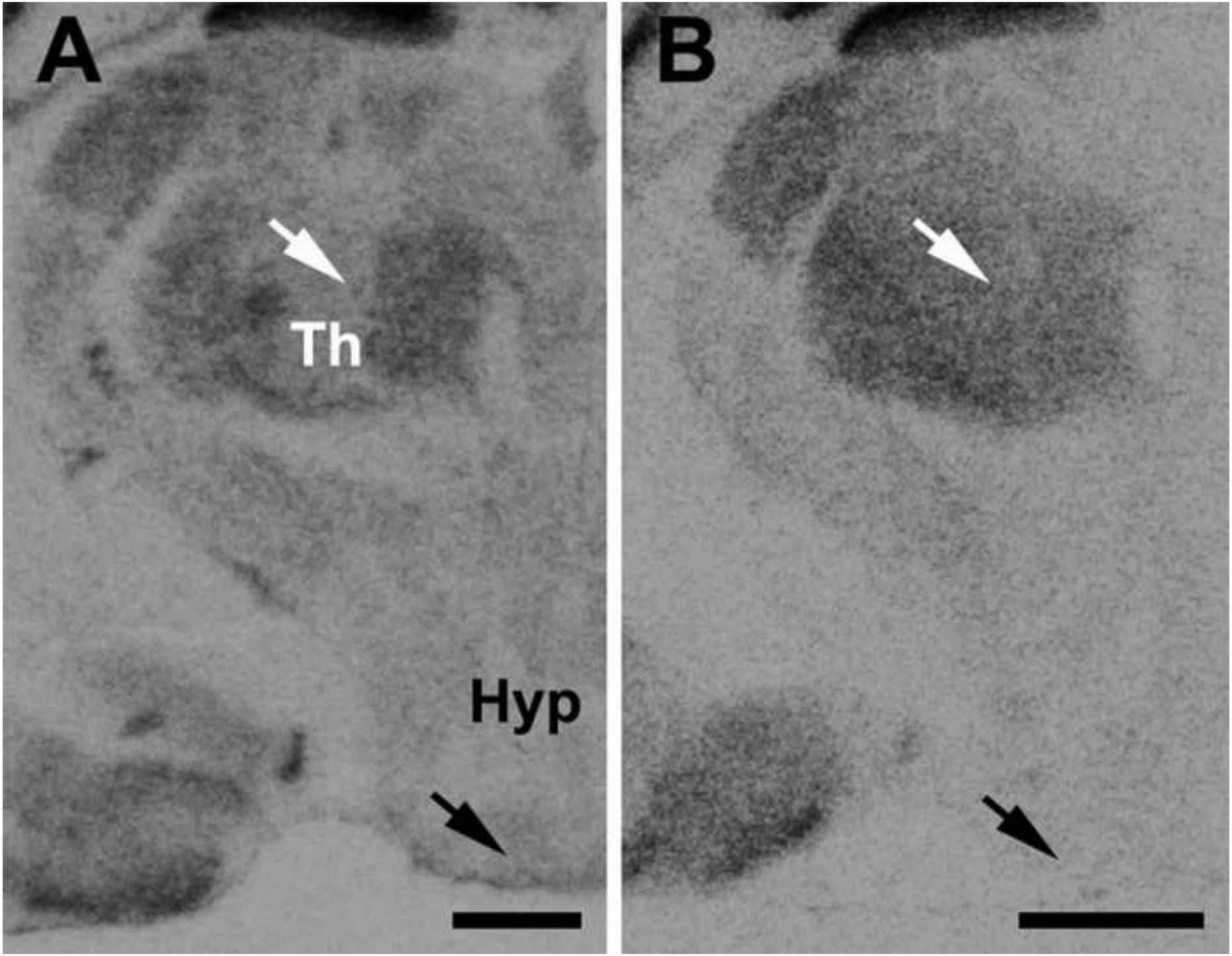

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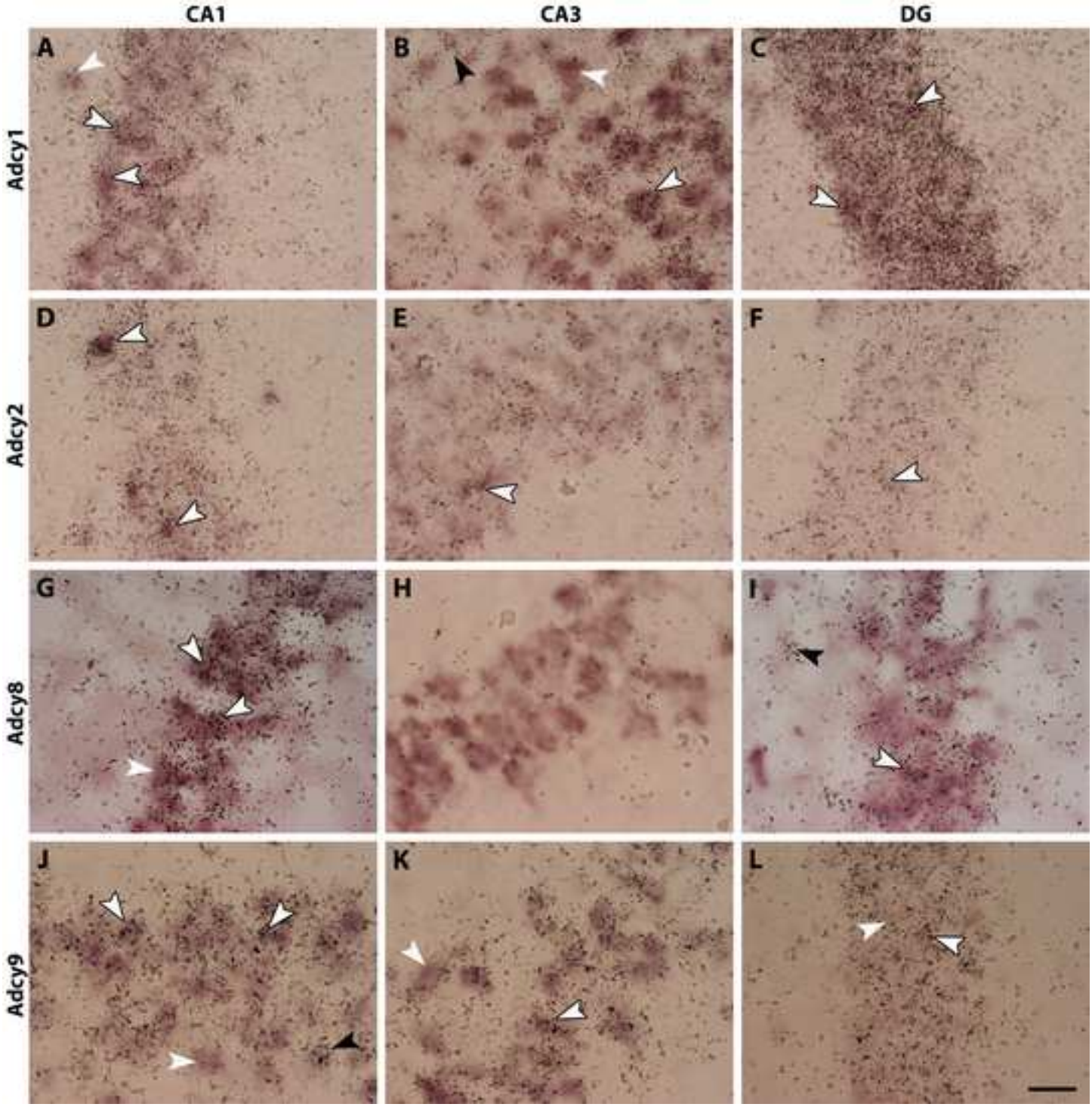

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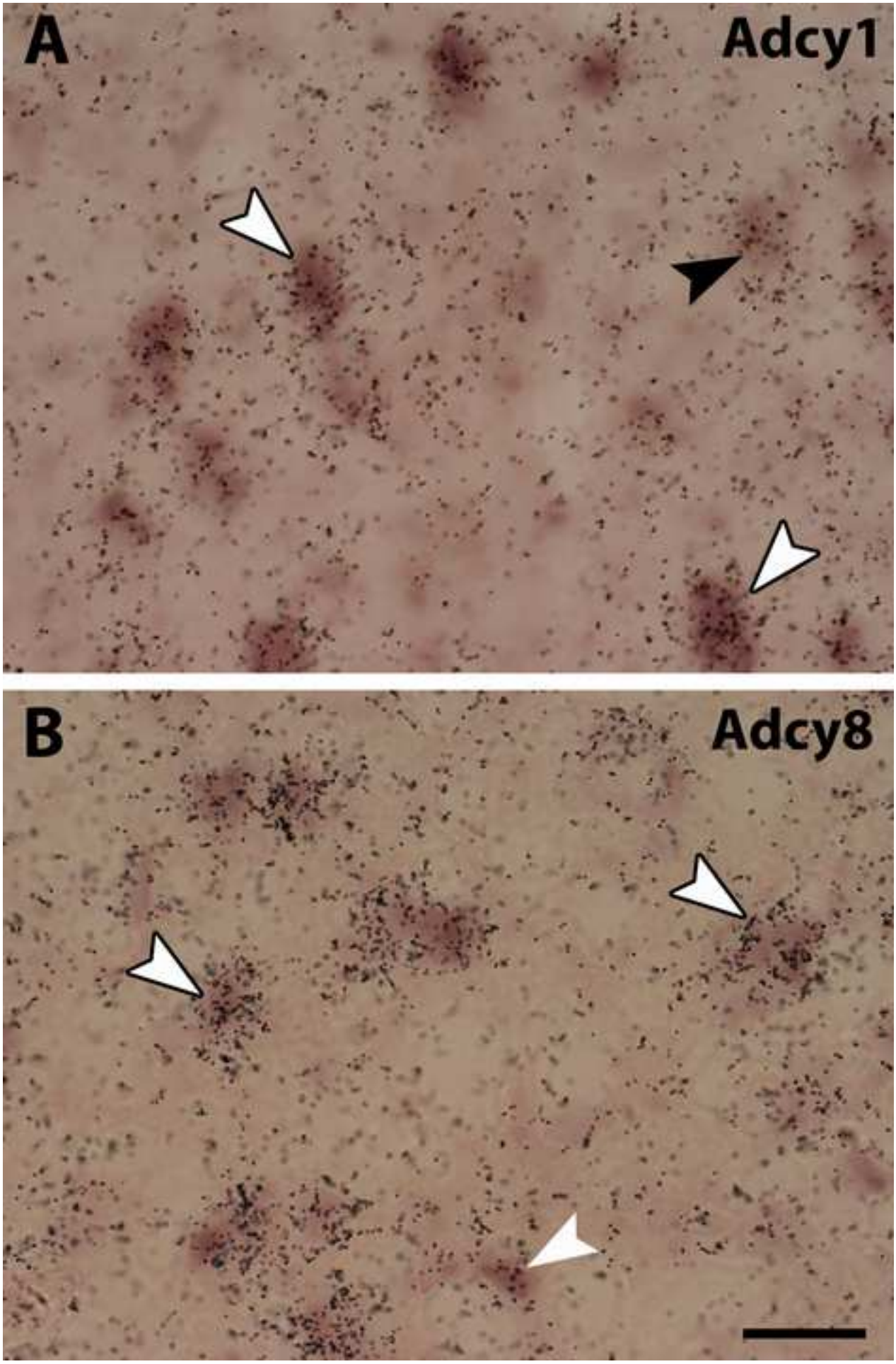

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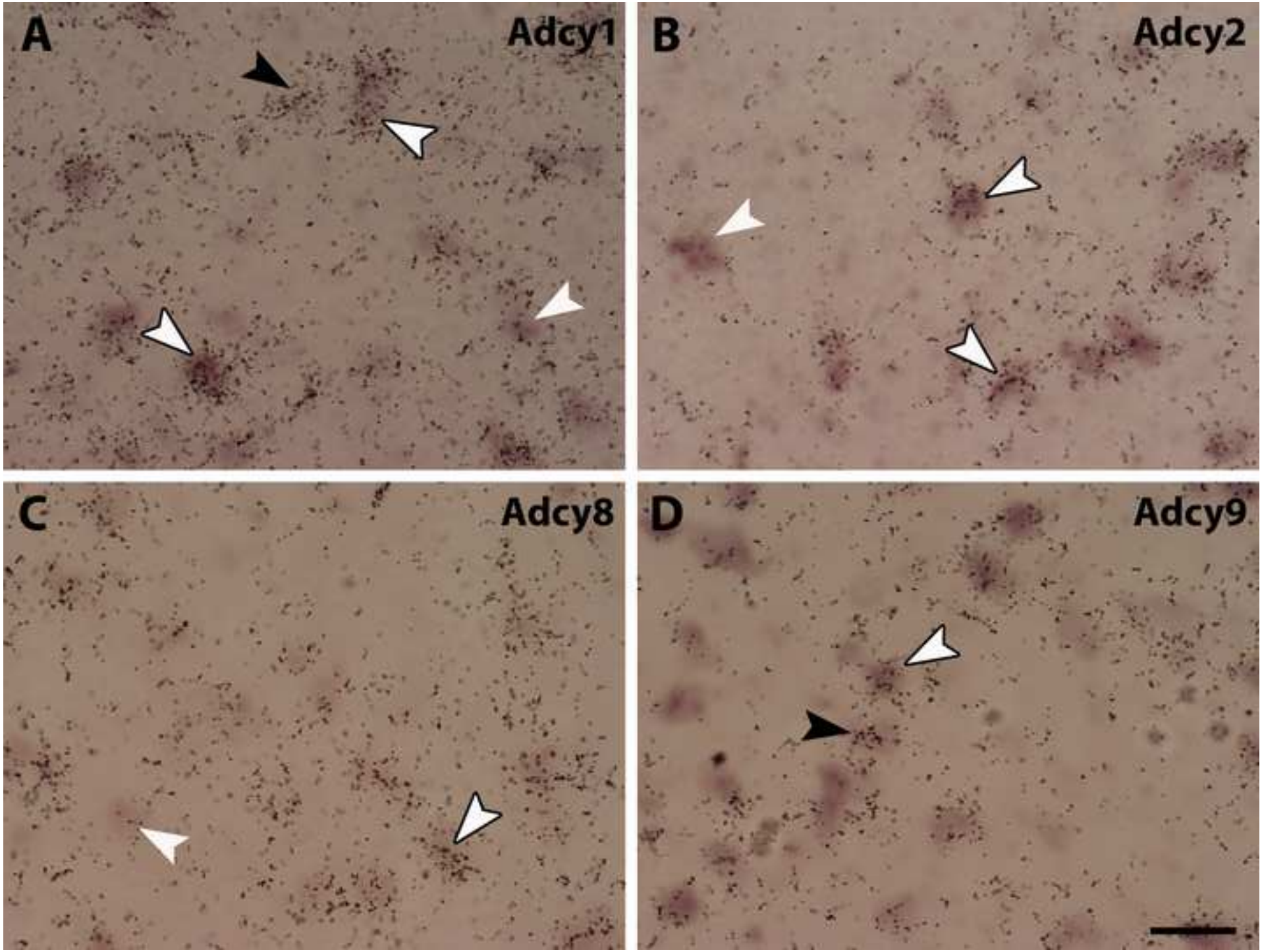

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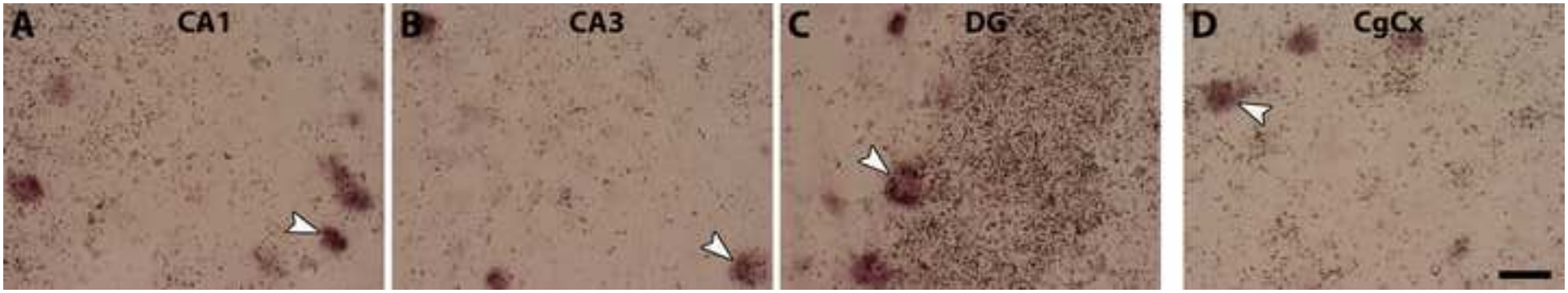

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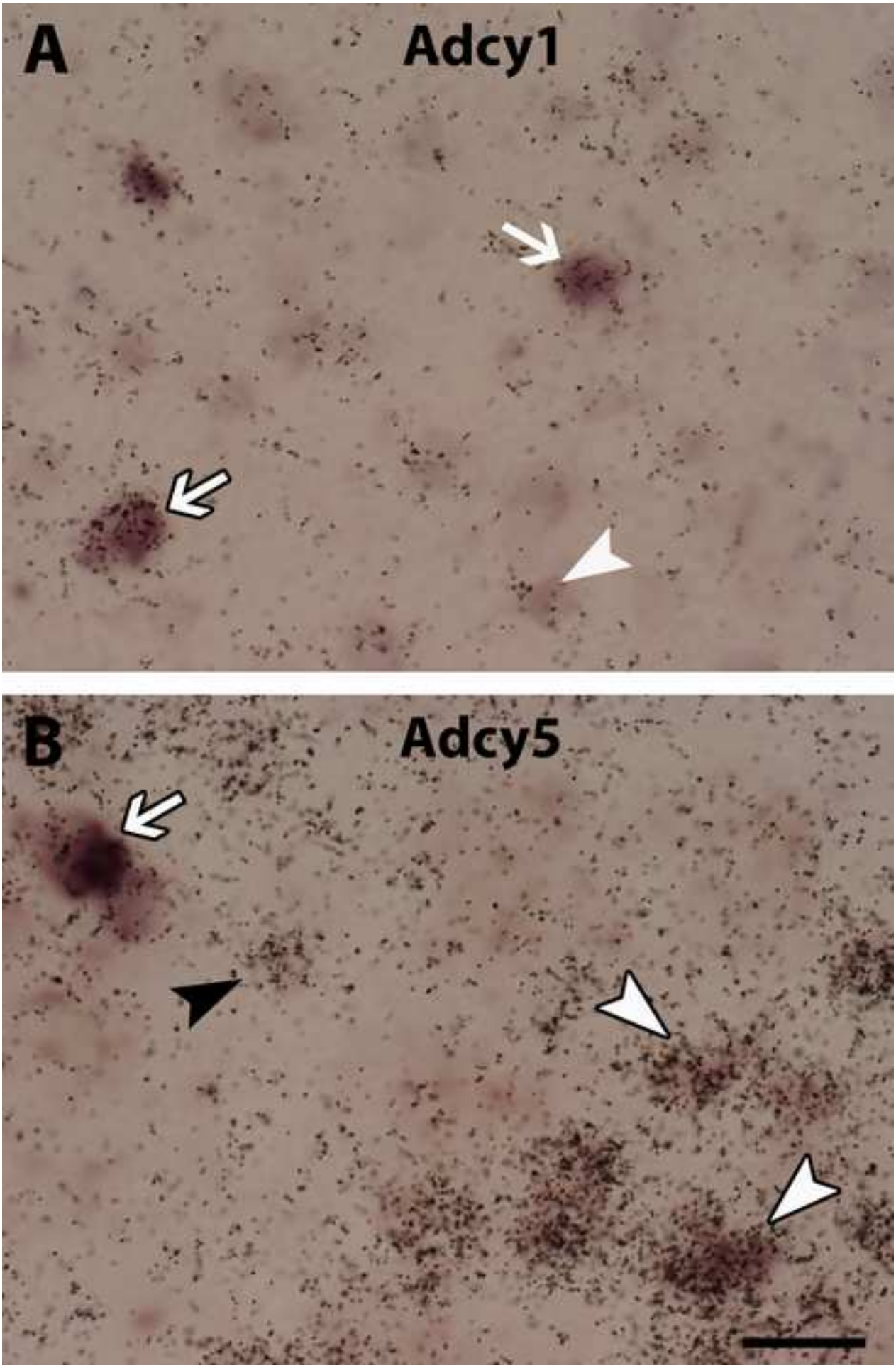

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