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Localization of Myocyte Enhancer Factor 2 in the Rodent Forebrain:

Regionally-Specific Cytoplasmic Expression of Myocyte Enhancer Factor 2A

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Abstract

The transcription factor myocyte enhancer factor 2 (MEF2) is expressed throughout the central nervous system, where four MEF2 isoforms play important roles in neuronal survival and differentiation and in synapse formation and maintenance. It is therefore somewhat surprising that there is a lack of detailed information on the localization of MEF2 isoforms in the mammalian brain. We have analyzed the regional, cellular, and subcellular expression of MEF2A and MEF2D in the rodent brain. These two MEF2 isoforms were co-expressed in virtually all neurons in the cortex and the striatum, but were not detected in astrocytes. MEF2A and MEF2D were localized to the nuclei of neurons in many forebrain areas, consistent with their roles as transcriptional regulators. However, in several subcortical sites we observed extensive cytoplasmic expression of MEF2A but not MEF2D. MEF2A was particularly enriched in processes of neurons in the lateral septum and bed nucleus of the stria terminalis, as well as in several other limbic sites, including the central amygdala and paraventricular nuclei of the hypothalamus and thalamus. Ultrastructural examination similarly revealed MEF2A-ir in axons and dendrites as well as nuclei of the lateral septum and bed nucleus of the stria terminalis neurons. This study demonstrates for the first time extensive cytoplasmic localization of a MEF2 transcription factor in the mammalian brain in vivo. The extranuclear localization of MEF2A suggests novel roles for MEF2A in specific neuronal populations.

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Keywords

bed nucleus of the stria terminalis; cortex; dendritic spine; myocyte enhancer factor; septum; transcription factor

1. INTRODUCTION

The transcription factor myocyte enhancer factor 2 (MEF2) was initially observed in muscle (Black and Olson, 1998). Since then it has become clear that MEF2 is also expressed in several other tissues, including cells of the immune and central nervous systems (Dodou, et al., 1995; Han, et al., 1997; Martin, et al., 1994; Youn, et al., 1999; Yu, et al., 1992). Gene duplication of a single ancestral gene found in invertebrates has led to the evolution of four distinct vertebrate MEF2 genes (MEF2A-D) (Shalizi, A. K. and Bonni, 2005). Tissue specific alternate mRNA splicing and posttranslational protein modifications result in additional MEF2 isoforms (Heidenreich and Linseman, 2004; Shalizi, A. K. and Bonni, 2005).

Transcripts encoding the four MEF2 proteins are expressed in the nervous system in a regionally- and developmentally-specific pattern (Edmondson, et al., 1994; Ikeshima, et al., 1995; Leifer, D., et al., 1994; Leifer, D, et al., 1993; Lin, et al., 1996; Lyons, et al., 1995; Mao, et al., 1999). Studies of MEF2 suggest a role for these transcription factors in neuronal differentiation and survival (Gaudilliere, et al., 2002; Lam and Chawla, 2007; Mao, et al., 1999; Mao and Wiedmann, 1999). MEF2C is expressed in postmitotic differentiating neurons, but not in proliferating precursor cells of primary cultures of the cortex, suggesting that MEF2 controls maturation of newly differentiated neurons (Mao, et al., 1999). Moreover, depolarization-induced MEF2 activation is necessary for the survival of differentiating neurons (Gaudilliere, et al., 2002; Mao, et al., 1999). Recently MEF2 transcription factors were implicated in synapse formation and maintenance. MEF2A/2D knockdown or activation in dissociated hippocampal neurons results in increased synapse formation or loss of synapses and dendritic spines, respectively (Flavell, et al., 2006). Interestingly, knockdown of MEF2A in developing cerebellar granule neurons in organotypic cultures or *in vivo* has the opposite effect, leading to decreased formation of synaptic structures (Shalizi, A., et al., 2006). Both Flavell et al. (2006) and Shalizi and colleagues (2006) found that the MEF2-mediated regulation of synapse number was affected by neuronal activity.

The information on the regional and cellular expression of MEF2 isoforms is limited (Leifer, D., et al., 1994; Leysen, et al., 2004; Lin, et al., 1996; Mao, et al., 1999). Because of the regionally-specific differences of MEF2 involvement in neuronal differentiation and survival, we examined the regional, cell-type specific, and subcellular expression of MEF2A and MEF2D in the rodent forebrain. We observed a regionally-specific pattern of cytoplasmic expression of MEF2A but not MEF2D.

2. RESULTS

Immunoblot analysis of MEF2A and MEF2D expression

In the cortex, striatum, hippocampus, and the lateral septum and bed nucleus of the stria terminalis (LS/BNST) immunoblot analysis revealed MEF2D as a single band of ~ 55 kD (Fig. 1B). MEF2A migrated as a doublet with a MW ~ 55 kD (Fig. 1A); the same protein doublet was observed using two anti-MEF2A antibodies generated against two different parts of the protein. The relative intensity of the upper band varied somewhat with different tissue preparations; this band was lost in tissue homogenates prepared in the absence of 2% sodium dodecyl sulfate, which inactivates phosphatases (data not shown) and is thus likely a phosphorylated form of MEF2A (Cox, et al., 2003). We occasionally observed a faint slower-

migrating MEF2A-ir band of ~ 85 kD that probably represents the sumoylated form of MEF2A (Riquelme, et al., 2006). No signal was observed in the absence of primary antibodies or after preadsorption of the primary antibody with the peptide immunogen.

Cellular MEF2A and MEF2D expression in the cortex and striatum

Immunohistochemical studies revealed a widespread distribution of MEF2A- and MEF2D-ir cells in the forebrain. Two different antibodies generated against different parts of the MEF2A protein yielded qualitatively identical staining patterns. No signal was observed when the primary antibodies were omitted or preadsorbed with the peptide immunogen.

We focused our initial efforts on assessing the localization of MEF2A and MEF2D in the neocortex and striatum. Both proteins were expressed in all cortical layers and throughout the striatum (Fig. 2A,B,D,E). In the cortex and the striatum the two MEF2 isoforms were almost exclusively localized to the cell nucleus (Fig. 3A,D; Fig.4), with rare cytosolic MEF2A-ir in neuronal processes observed in the medial (periventricular) striatum (Fig. 3E). Double labeling experiments (Fig. 4A,B,D,E) revealed that essentially all cortical and striatal neurons contain both MEF2A and MEF2D, although relative expression levels vary (Fig. 4C,F).

Staining of cortical and striatal tissue with anti-MEF2A antibodies and DAPI revealed an overlap of MEF2A-ir and DAPI in the larger (presumably neuronal) nuclei, but not in the smaller (glial) nuclei (Fig. 3C,F), suggesting that MEF2A is not present in glial cells. Consistent with this impression, we found that in both the cortex and the striatum MEF2A-ir was found in cells expressing the neuronal marker NeuN (Fig. 3A,D) but not in GFAP-ir astrocytes (Fig. 3B,E). Because MEF2A and MEF2D are co-expressed one can infer that ME2D is also expressed in neurons but absent in astrocytes.

Although gross examination suggested an enrichment of MEF2A and MEF2D in the superficial relative to deep layers of the rat (Fig. 2A,D) and mouse (data not shown) cortex, double labeling with antibodies against MEF2A and NeuN revealed that almost all cortical neurons expressed MEF2A, regardless of laminar localization (Fig. 3A). This observation suggests that MEF2A and MEF2D are present in both projection neurons and interneurons. We assessed the presence of MEF2A-ir in three largely non-overlapping populations of cortical interneurons, defined on the basis of expression of the calcium binding proteins parvalbumin, calbindin or calretinin (Kubota, et al., 1994). MEF2A was detectable in the great majority of but not all cortical interneurons (Fig. 5). The same observation was made for MEF2D (data not shown).

Extranuclear expression of MEF2A

As noted above, only rarely were MEF2A-ir cellular processes seen in the striatum and neocortex. In contrast, extensive cytoplasmic MEF2A-ir was observed in neuronal processes in a variety of forebrain sites (Table 1). We examined extranuclear MEF2A-ir in greater detail in the LS/BNST, in which MEF2A-ir fibers are particularly abundant (Fig. 2C). MEF2A-ir processes were observed in both the adult rat (Fig. 6A) and to a lesser degree in the E20 embryo (data not shown). MEF2A-ir processes were also abundant in the mouse LS/BNST (Fig. 6B). The perisomatic and peridendritic puncta seen at higher magnification suggest that MEF2A is primarily localized to axons and axon terminals (Fig. 6C). However, the density of MEF2A-ir processes in the LS/BNST was so great that it was not possible at the light microscopic level to determine if MEF2A-ir was localized to axons, dendrites, or both.

Ultrastructural localization of MEF2A

We examined the subcellular localization of MEF2A in the LS/BNST in more detail using preembedding immunoelectron microscopy. Although MEF2A-ir was seen in neuronal nuclei, it was most prominent in axon terminals (Fig. 7A); MEF2A-ir was also seen in preterminal

portions of myelinated (Fig. 7B) and unmyelinated axons (Fig. 7C). In addition, MEF2A-ir was localized to dendritic spines (Fig. 7D) and shafts (Fig. 7E). MEF2A-ir was found in symmetric and asymmetric synapses, with the former typically being seen in association with proximal dendrites (Fig. 8A) or neuronal cell bodies (Fig. 8B,C), and the latter associated with dendritic spines (Fig. 7D).

3. DISCUSSION

MEF2A and MEF2D are widely expressed in the rat forebrain, where they are co-expressed in the large majority of the neurons and are mostly localized to neuronal nuclei. However, we show here for the first time that in several regions of the mammalian brain, MEF2A is abundantly present in the neuronal cytoplasm, where it is most prominently localized to neuronal processes and their terminals. The cytoplasmic expression of MEF2A was particularly prominent in limbic regions of the rodent forebrain. This observation suggests that the function of MEF2A may vary across different forebrain regions.

Regional and cellular MEF2A and MEF2D expression in the rat brain

In the rat cortex MEF2A and MEF2D were co-expressed in almost all neurons. Gross examination of MEF2A- and MEF2D-ir suggested enrichment of these proteins in the superficial layers of the rat and mouse cortex, an observation previously reported in the feline cortex (Leysen et al., 2004). However, we found that the two MEF2 isoforms were invariably expressed in NeuN-positive cells. NeuN is expressed in the great majority of mammalian neurons, with only a few neurons lacking detectable levels of this protein (Mullen, et al., 1992). Thus, the apparent greater density of MEF2A/D-ir cells in the superficial layers of the rat cortex probably reflects the greater density of neurons in layers II/III, although a subjective appearance of greater density may also be conveyed by increased levels of MEF2A in neurons of supragranular relative to infragranular layers. MEF2A also appeared to be enriched in the superficial layers of the mouse cortex, reflecting the concordance of regional and neuronal MEF2A localization in rat and mouse brain.

MEF2A and MEF2D were co-expressed in virtually all striatal neurons, including dorsal striatal and ventral striatal (nucleus accumbens) territories. The colocalization of MEF2A and MEF2D in the striatal complex is in agreement with previous data suggesting that these two MEF2 isoforms bind to DNA as heterodimers in striatal (presumptive medium spiny) neurons (Pulipparacharuvil, et al., 2008). The ubiquitous expression of MEF2A and MEF2D stands in contrast to the expression pattern of MEF2C, which is present in distinct subsets of neurons (Leifer, D., et al., 1994; Leifer, D, et al., 1993; Speliotes, et al., 1996).

We found that MEF2A- and MEF2D-ir was present in neurons but absent in astrocytes, consistent with previous reports indicating that MEF2A is restricted to neurons in the cat and mouse cortex and the cerebellum (Ikeshima, et al., 1995; Leysen, et al., 2004). The predominantly neuronal expression of the MEF2 transcription factors in the mammalian brain is particularly interesting in view of the role that these proteins have been suggested to play in synapse formation and maintenance (Flavell, et al., 2006; Shalizi, A., et al., 2006).

Extranuclear localization of MEF2A

We were surprised to observe a previously unreported extranuclear localization of MEF2A-ir, which manifested itself most prominently in axons and axon terminals but was also seen in dendritic shafts and spines.

Several findings indicate that this cytoplasmic MEF2A-ir is specific and represents MEF2A. First, omission of the primary antibody or preadsorption of the MEF2A antibody with the

peptide immunogen resulted in a complete lack of staining on rat brain sections and immunoblots. Second, we obtained identical MEF2A staining with two different antibodies generated against different peptide sequences of MEF2A. Finally, immunoblots showed the same protein bands, at the predicted masses, in the LS/BNST (where MEF2A-ir processes are prominent) and the cortex and the striatum (sites in which almost no extranuclear MEF2A-ir is seen). These data are consistent with the conclusion that the MEF2A-ir seen in neuronal processes represents endogenous MEF2A. Moreover, we observed the same pattern of MEF2A-ir in rat and mouse brains, suggesting that the localization of MEF2A to axons and dendrites is not a species specific occurrence.

We never observed MEF2D outside of the nucleus in any brain area examined. However, in cell cultures MEF2D has been reported to be in the cytoplasm of differentiating neuronal precursor cells (Lam and Chawla, 2007), and cytoplasmic accumulation of MEFD has recently been reported in a mouse dopaminergic progenitor cell line after inhibition of chaperone-mediated autophagy (Yang, et al., 2009). The discrepancy in the location of MEF2D observed in brain sections and in cultured neuronal cells may reflect differences in MEF2D trafficking in neurons in vitro and in vivo.

Cells with MEF2A-ir processes are clustered in limbic areas, including the LS/BNST, the central nucleus of the amygdala, and the paraventricular nucleus of the hypothalamus. Strikingly absent, however, were MEF2A-ir processes in the hippocampus. “Mesolimbic” areas abutting the limbic sites, including the septal pole of the nucleus accumbens, were also relatively heavily labeled. Finally, neurons with MEF2A-ir processes could be observed in some intralaminar thalamic nuclei, especially the thalamic paraventricular nucleus. Thus, neurons with MEF2A-ir processes are clustered in subcortical regions with important roles in autonomic function (Kerman, 2008; Loewy, 1991) and in motivation and reward (Deutch, et al., 1998; Heimer, 2003; Koob, 2008).

Conclusions

The functional significance of extranuclear MEF2A is unclear. The localization of most transcription factors is nuclear, with rapid importing of the proteins into the nucleus after cytosolic translation. MEF2D expressed in a dopaminergic progenitor cell line has been suggested to be shuttled out of the nucleus into the cytoplasm for lysosomal degradation (Yang, et al., 2009). We did not observe MEF2A-ir in lysosomes or multivesicular bodies, and thus the axonal and axon terminal localization of MEF2A, where lysosomes are not typically found, suggests a different function for cytoplasmic MEF2A.

A number of examples of extranuclear transcription factors have been reported, including Stat1 (Gautron, et al., 2003), Elk-1 (Barrett, et al., 2006), and Engrailed1 (Di Nardo, et al., 2007). Some transcription factors are found in mitochondria, an organelle abundant in axonal terminals, where they regulate mitochondrial maintenance and proliferation (Scarpulla, 2002); an example is Elk-1, which associates with the mitochondrial permeability transition pore. However, we did not observe MEF2A labeling of mitochondria.

MEF2A in hippocampal and cerebellar neurons regulates the formation and maintenance of spines and the associated excitatory synapses by modulating the expression of synapse specific genes (Flavell, et al., 2006; Shalizi, A., et al., 2006). These observations raise the possibility that MEF2A plays a role in the formation of excitatory synapses in areas such as the hippocampus and the cerebellum, where it is localized exclusively to the nuclei, but may play an additional role in areas such as the LS/BNST, amygdala, and paraventricular hypothalamus, where it is localized to axons and dendrites as well as nuclei and is primarily associated with symmetric (inhibitory) synapses.

Dysregulation of transcription factor trafficking or signaling has been linked to diverse neuropsychiatric disorders, from substance abuse to neurodegenerative disorders such as amyotrophic lateral sclerosis (Chu, et al., 2007; Pulipparacharuvil, et al., 2008). Indirect evidence suggests that MEF2 proteins may be involved in such disorders. For example, some MEF2 isoforms are downstream targets of key intracellular signaling proteins such as calcium-calmodulin dependent protein kinase II (Zhang, et al., 2007) that regulate dendritic remodeling (Okamoto, et al., 2007), which is seen in both Parkinson's disease and psychostimulant abuse (Robinson and Kolb, 2004; Zaja-Milatovic, et al., 2005). In addition, MEF2 has been shown to regulate cocaine-induced changes in dendritic spines (Pulipparacharuvil et al., 2008). At this point the biological function of MEF2A in extranuclear sites, including axons and dendrites, is not known. Determination of the mechanisms that control MEF2A trafficking will be required to unravel the roles of this transcription factor in the different cellular compartments.

4. EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats (Harlan; Indianapolis, IN) were group-housed on a 12:12 light:dark cycle with food and water available *ad libitum*. All experiments were conducted in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals and under the oversight of the institutional Animal Care and Use Committees.

Antibodies

Two different MEF2A antibodies were used. The first is an affinity-purified rabbit polyclonal antibody that was generated against a C-terminal peptide of human MEF2A corresponding to amino acids 487-507 (Li, et al., 2001) (sc-131; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specificity of the antibody was assessed by Western blotting and preadsorption of the antiserum with the immunogen peptide (sc313P; Santa Cruz Biotechnology) (see results). The other MEF2A antibody used was a rabbit polyclonal raised amino acids 272-491 of human MEF2A (ab32866; Abcam, Cambridge, MA). The specificity of this antibody was confirmed by Western blotting. The MEF2D antibody was a mouse monoclonal antibody raised against amino acids 346-511 of the mouse MEF2D protein (610774; BD Biosciences, San Jose, CA); again, the specificity of this antibody was confirmed by immunoblotting (see results).

Other antibodies that were used included a mouse anti-NeuN antibody made against purified cell nuclei from mouse brain (MAB377; Millipore, Temecula, CA), mouse anti-glial fibrillary acidic protein (GFAP) raised against purified bovine GFAP (MAB360; Millipore), mouse anti-parvalbumin raised against purified frog muscle parvalbumin (P3088; Sigma-Aldrich Co., St. Louis, MO), rabbit anti-calbindin produced against recombinant rat calbindin-D-28k (CB-38; SWant, Bellinzona, Switzerland), mouse anti-calbindin-D-28K made against purified bovine kidney calbindin-D-28K (C9848; Sigma-Aldrich), goat anti-calretinin raised against rat calretinin (AB1550; Millipore), and a mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody generated against GAPDH from rabbit muscle (MAB374; Millipore).

Immunoblot analyses

Animals were decapitated under isofluorane anesthesia and the cerebral cortex, striatum, hippocampus, and the lateral septum and bed nucleus of the stria terminalis (LS/BNST) dissected from coronal slices. Samples were sonicated in 2% sodium dodecyl sulfate, and protein concentrations determined with a Lowry-based protein microassay (BioRad, Hercules, CA). Total protein (10 to 20 μg) was separated by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (Laemmli, 1970) and transferred to a PVDF membrane (Immobilon-P, Millipore, Temecula, CA). The membranes were incubated in Tris-buffered saline containing 4% non-fat dry milk and 0.2% Tween 20 for 3 hours and then incubated overnight at 4°C in

the following primary antibodies diluted in the same buffer: rabbit anti-MEF2A (1:100, Santa Cruz Biotechnology and 1:500, Abcam), mouse-anti-MEF2D (1:4000), and mouse anti-GAPDH antibodies (1:4,000,000). After washing the membranes were incubated in secondary HRP-conjugated anti-mouse- or anti-rabbit antibodies (1:10,000; Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature or overnight at 4°C. The signal was developed by chemiluminescence ("Western Lightning", PerkinElmer Life Sciences, Boston, MA) and visualized on a HyBlot CL film (Denville Scientific, Inc., Metuchen, NJ).

Immunohistochemistry

Rats or mice were deeply anesthetized with isofluorane and perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde (PFA) in the same buffer. Brains were removed and postfixed overnight, cryoprotected in 0.1M phosphate with 30% sucrose, and 42 μm coronal sections were cut on a freezing microtome. The sections were stored in 0.1M phosphate buffer containing 30% sucrose and 30% ethylene glycol at -20°C. Immunofluorescence and immunoperoxidase methods were used to localize MEF2 proteins in the forebrain. For immunofluorescent staining free-floating sections were first incubated in 50 mM Tris-buffered saline containing 4% horse serum and 0.2% Triton-X-100 (TBS^+) for 30 min and then incubated overnight at room temperature in one the following antibodies: rabbit anti-MEF2A (1:200, Santa Cruz Biotechnology or 1:1000, Abcam), mouse-anti-MEF2D (1:4000), mouse anti-NeuN (1:200); mouse anti-glial fibrillary acidic protein (GFAP) (1:2000), mouse anti-parvalbumin (1:1500), rabbit anti-calbindin (1:1000), mouse anti-calbindin (1:2000), or goat anti-calretinin (1:1000) in TBS^+. The sections were subsequently incubated in secondary antibodies conjugated to either Alexa-488 (1:250; Invitrogen, Carlsbad, CA) or Cy3 (1:1000; Jackson ImmunoResearch) in TBS^+, then washed and mounted in DPX.

For immunoperoxidase detection the sections were incubated in methanolic peroxide for 10 minutes, rinsed, and incubated in TBS^+ for 30 minutes, followed by incubation overnight at room temperature in mouse anti-MEF2D (1:12,000) or rabbit anti-MEF2A (1:600, Santa Cruz or 1:3000, Abcam) diluted in TBS^+. Sections were subsequently incubated in biotinylated secondary antibodies (1:1000; Jackson ImmunoResearch) in TBS^+ for 90 min and then in peroxidase-conjugated streptavidin (1:1600; Jackson ImmunoResearch) for 60 min. The signal was developed in a solution containing 0.025% diaminobenzidine (DAB), 0.4% nickel ammonium sulfate, 0.04% cobalt chloride, and 0.001% hydrogen peroxide. Control procedures included omission of primary antibodies and preadsorption of the MEF2A antiserum with the peptide immunogen. Brain regions were identified and labeled using the brain atlas and nomenclature of Paxinos and Watson (Paxinos and Watson, 2007).

Ultrastructural studies

Rats were perfused with 4% PFA, 0.2% glutaraldehyde, and 0.2% picric acid in PBS, and the brains blocked and postfixed in 4% PFA for 4 hours. Sections were cut on a vibrating microtome at 50 μm. Immunoperoxidase labeling was performed as previously described (Muly, et al., 2003). Briefly, sections were incubated in blocking solution (3% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% lysine in 10 mM phosphate buffered saline, pH 7.4) for 1 hour prior to incubation in rabbit anti-MEF2A (1:600; Santa Cruz) for 18-36 hours at 4°C. The sections were subsequently incubated in biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 60 min at room temperature, and then placed in Vectastain Elite ABC reagent (Vector Laboratories) for one hour. The signal was developed using DAB as the chromagen. Sections were postfixed in osmium tetroxide, dehydrated, contrasted en bloc with uranyl acetate, and flat embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). Blocks from LS/BNST were prepared and ultrathin sections cut and collected on pioloform-coated slot grids and counterstained with lead citrate. Ultrathin sections were examined with a Zeiss EM-10 electron microscope and immunoreactive elements imaged

at a magnification of 33,500 using a Dualvision cooled CCD camera (1300 × 1030 pixels) and Digital Micrograph software (Gatan, Inc., Pleasanton, CA).

Immunoreactive profiles were identified using ultrastructural criteria (Peters, et al., 1991). We identified spines based on size (0.3-1.5 μm in diameter), presence of spine apparatus, absence of mitochondria or microtubules, and in some cases presence of asymmetric synaptic contacts. Dendrites were identified by their greater size (0.5 μm or more in diameter) and the presence of microtubules, mitochondria, and in some cases synaptic contacts. Axon terminals were characterized by the presence of numerous vesicles, mitochondria, and occasionally a presynaptic specialization. Preterminal, unmyelinated axons were identified by their small size (0.1- 0.3 μm in diameter); regular, round shape; and occasional presence of synaptic vesicles or neurofilaments. Glial profiles were identified based on their characteristic shape, which appears to fill in the space between other, nearby profiles, and a relatively clear cytoplasm, which occasionally contained numerous filaments. Cell bodies were identified by their content of ribosomes, rough endoplasmic reticulum, and occasionally the presence of a nucleus, golgi apparatus or other organelles in the examined section.

No MEF2A signal was observed in sections that were processed in the absence of the primary antibody.

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Abbreviations

BNST, bed nucleus of the stria terminalis; DAB, diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; -ir, immunoreactivity; LS/BNST, lateral septum and bed nucleus of the stria terminalis; MEF, myocyte enhancer factor; PFA, paraformaldehyde; TBS^{+}, Tris-buffered saline containing 4% horse serum and 0.2% Triton X-100.

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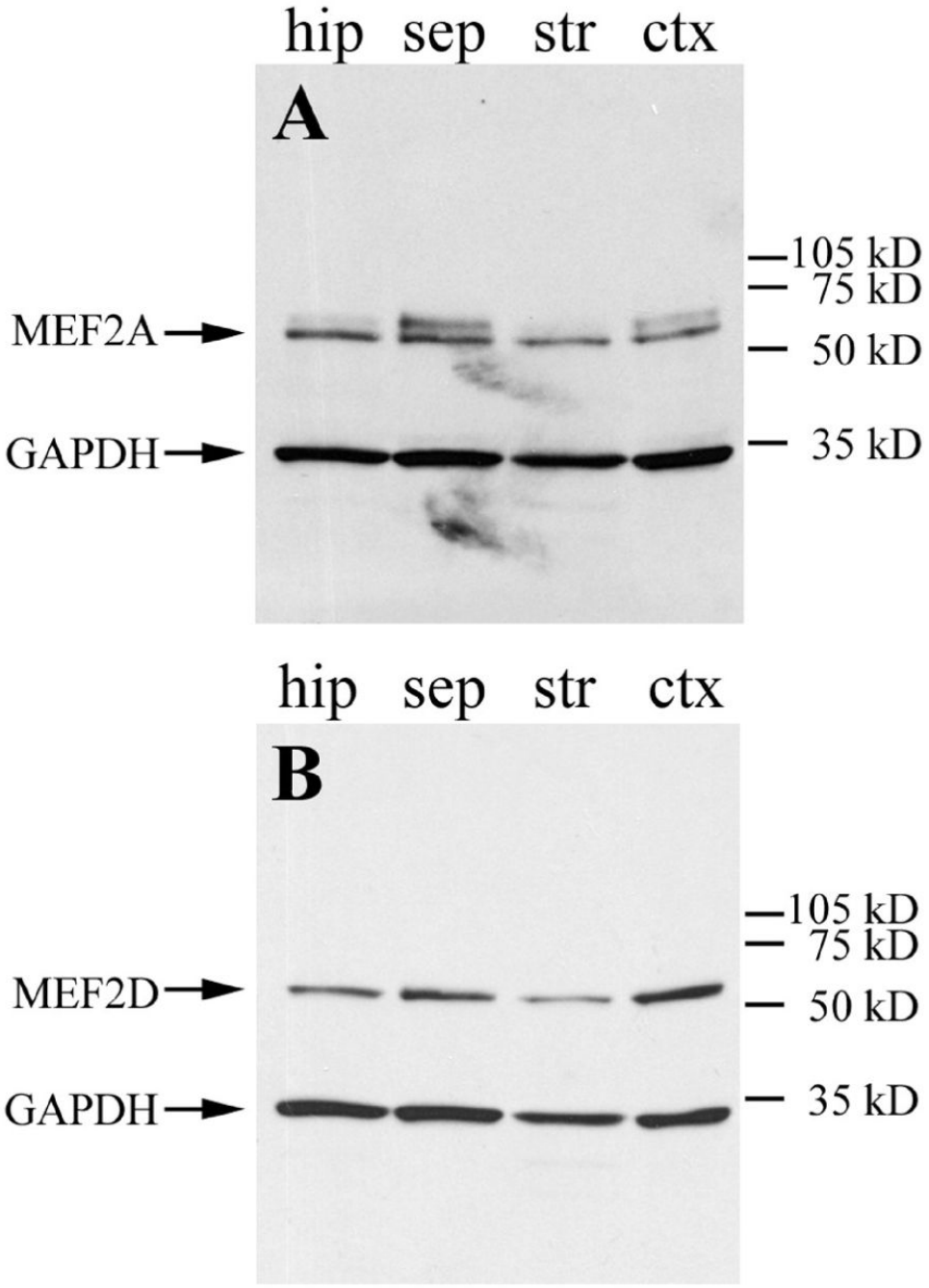

Figure 1.

Immunoblot analysis of MEF2A and MEF2D in the rat forebrain. Incubation with an antibody against MEF2A revealed a protein doublet of ~ 55 kD in all tissues examined (**A**), while MEF2D migrated as a single band at around 55 kD (**B**); GAPDH was included as a loading control. Molecular weight standards are indicated at the right. (hip, hippocampus; sep, septum; str, striatum; ctx, cortex).

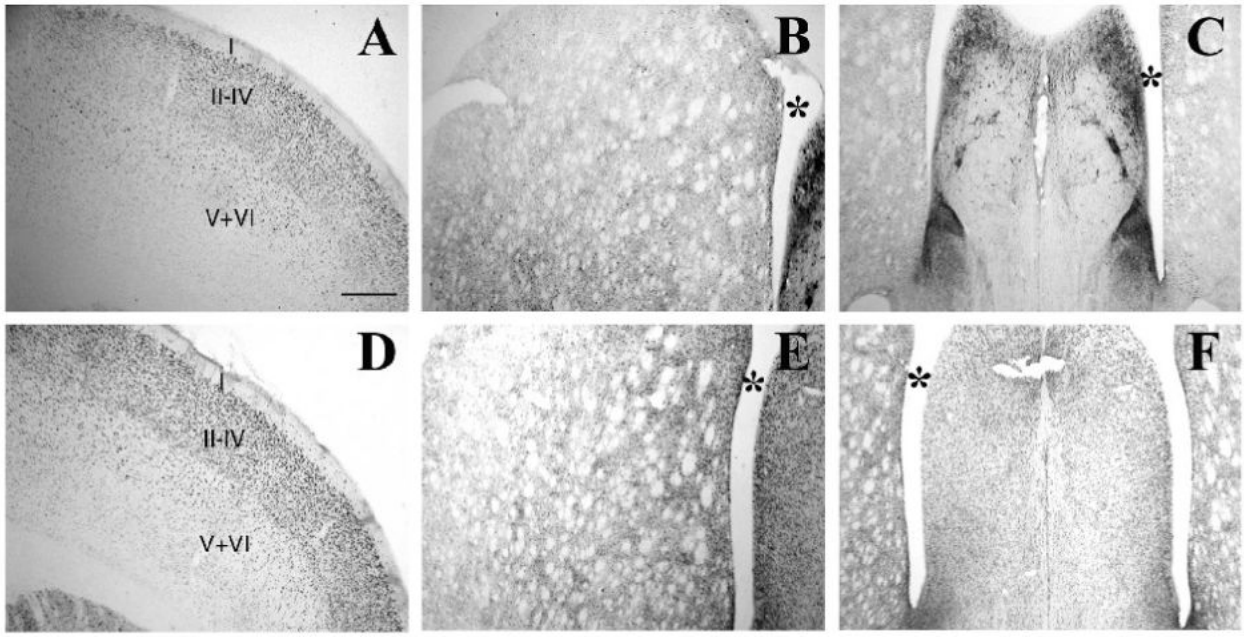

Figure 2.

Immunhistochemical analysis of MEF2A and MEF2D in the rat forebrain. MEF2A (**A-C**) and MEF2D (**D-F**) expression were analyzed in the cortex (**A,D**), striatum (**B,E**), and the septum (**C,F**) of the adult rat by immunoperoxidase staining. In the cortex MEF2A- (**A**) and MEF2D-ir (**D**) were seen in nuclei of neurons across all cortical lamina. MEF2A- (**B**) and MEF2D-ir (**E**) nuclei were also seen throughout the striatum. In contrast, although MEF2D-ir is localized to nuclei in the septum (**F**), extensive MEF2A-ir processes are present in the LS/BNST (**C**). (*, lateral ventricle, cortical layers are indicated by Roman numerals). Scale bar = 500 μm.

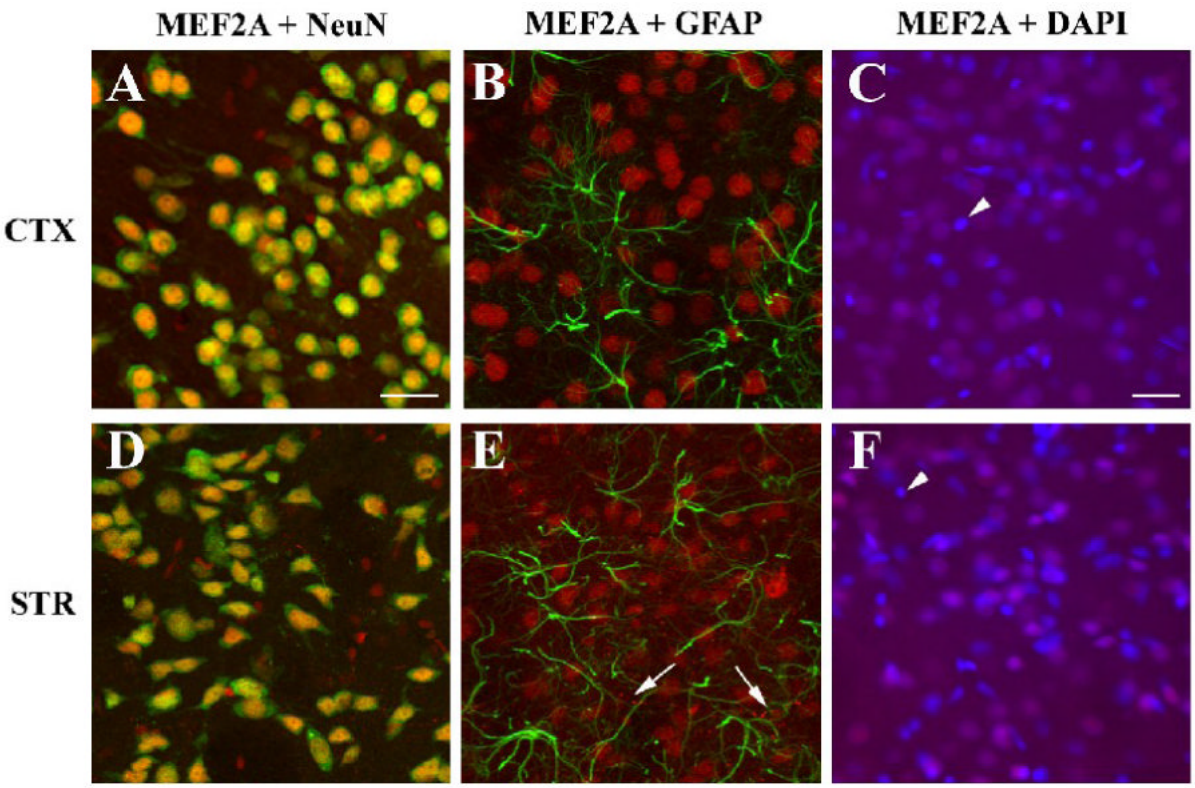

Figure 3.

MEF2A is expressed in neurons but not astrocytes. Cell type specific expression of MEF2A was assessed in the cortex (**A-C**) and striatum (**D-E**). MEF2A-ir (red) was almost always localized to nuclei in the cortex and striatum, although rare MEF2A-ir fiber-like structures can be seen in the medial (periventricular) striatum (**E**, arrows). Double-labeling experiments with antibodies against MEF2A (red) and the neuronal marker NeuN (green) reveals that virtually all NeuN positive cells express MEF2A in the cortex (**A**) and the striatum (**B**). In contrast, MEF2A-ir (red) does not colocalize with astrocytes labeled with GFAP (green) in the cortex (**B**) or the striatum (**E**). Counter-staining cortical (**C**) or striatal (**F**) sections with the nuclear stain DAPI (blue) reveals that virtually all large nuclei characteristic of neurons are MEF2A-positive (red), whereas in the smaller nuclei, typically associated with glial cells, MEF2A-ir is absent (**C,F** arrow head). Scale bar = 25 μm in A also applies to B, D, E; scale bar = 25 μm in C also applies to F.

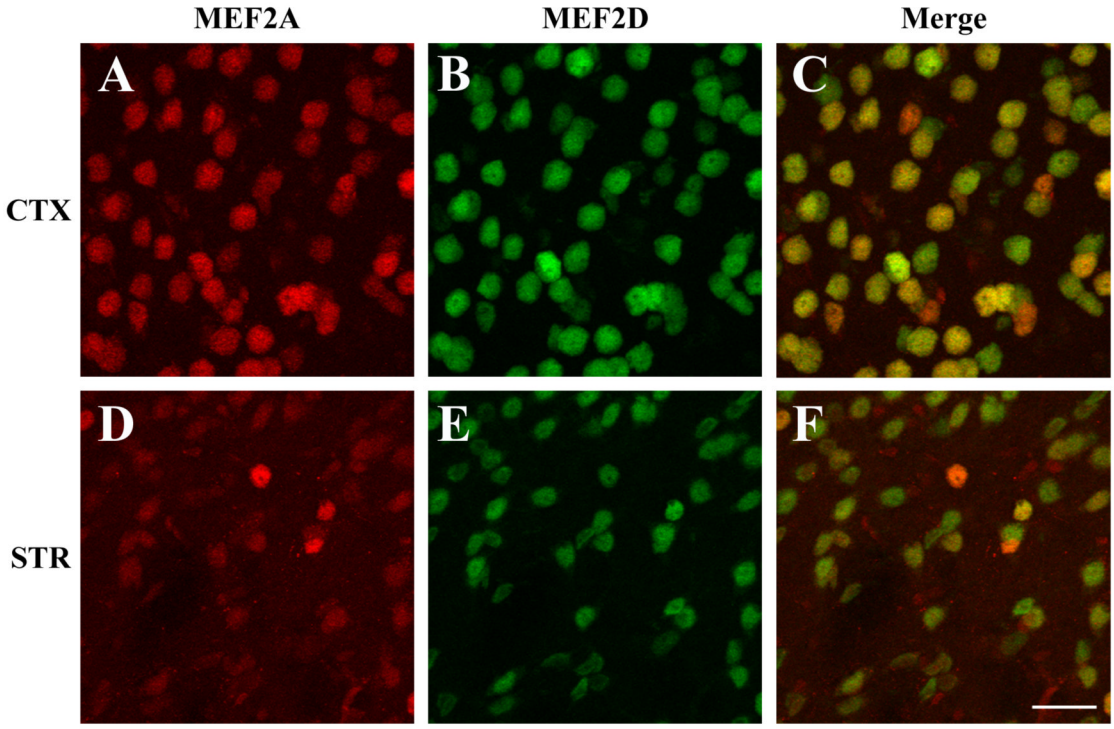

Figure 4.

MEF2A and MEF2D are colocalized in cortical and striatal neurons. Cortical (**A-C**) and striatal (**D-E**) sections were incubated with MEF2A (red) and MEF2D (green) antibodies. The merged images (**C, F**) demonstrate that MEF2A and MEF2D are co-expressed in virtually all cortical and striatal neurons, although the relative abundance of the two isoforms may vary somewhat, as reflected by the different shades of yellow. Scale bar = 25 μm.

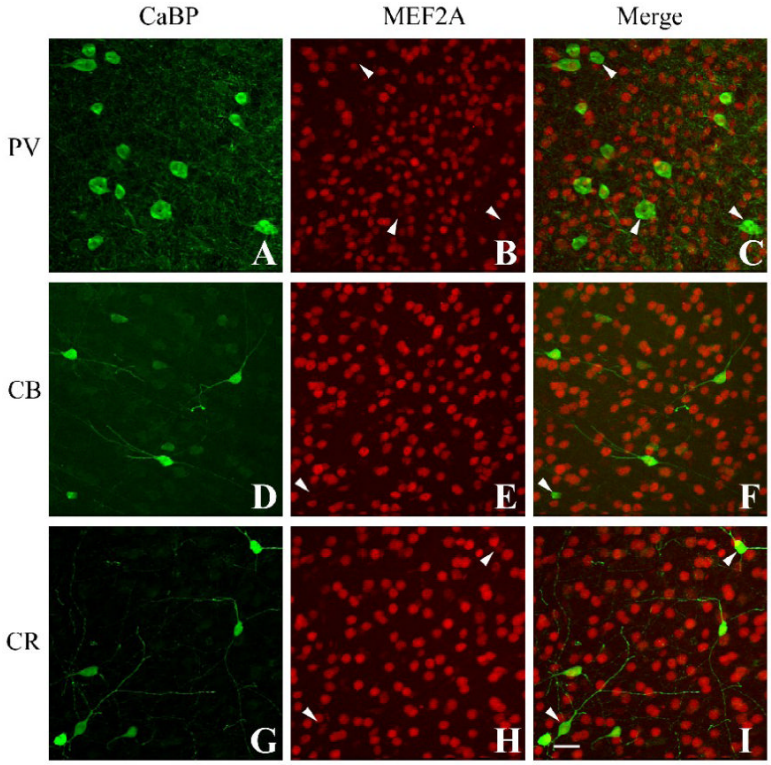

Figure 5.
Analysis of MEF2A expression in cortical interneurons. Cortical sections were stained for MEF2A (red) and one of three calcium binding proteins (CaBP) (green) that mark non-overlapping populations of cortical interneurons: parvalbumin (PV) (**A-C**), calbindin (CB) (**D-F**) or calretinin (CR) (**G-I**). Many, but not all (arrow heads) interneurons express MEF2A. Scale bar = 25 μm.

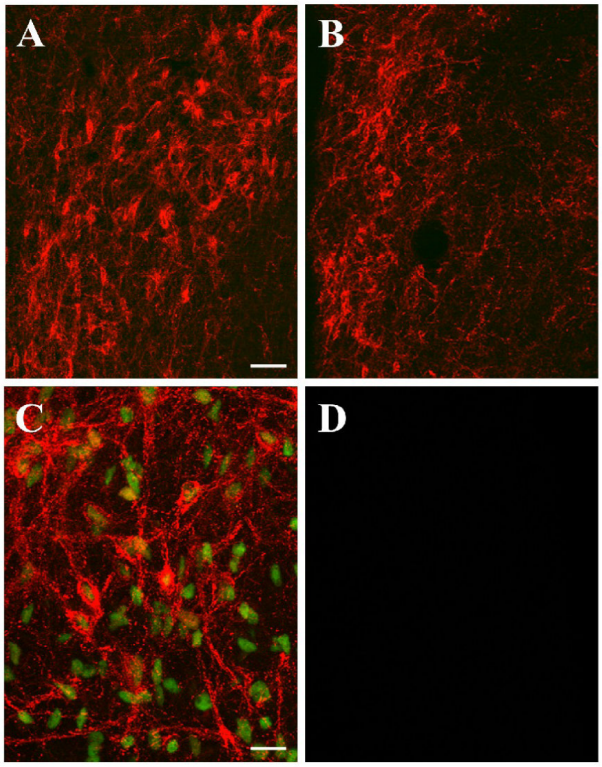

Figure 6.

Extranuclear MEF2A-ir in neurons of the LS/BNST. Abundant MEF2A-ir fiber-like structures can be clearly seen in the LS/BNST of adult rats (**A**) and mice (**B**). Higher magnification images reveal a punctate perisomatic and neuritic MEF2A staining pattern (**C**). MEF2D (green) is exclusively seen in nuclei (**C**). Preincubation of the MEF2A antibody with the peptide that served as the immunogen obliterated the signal (**D**). Scale bar = 50 μm in A,B and D; scale bar = 25 μm in C.

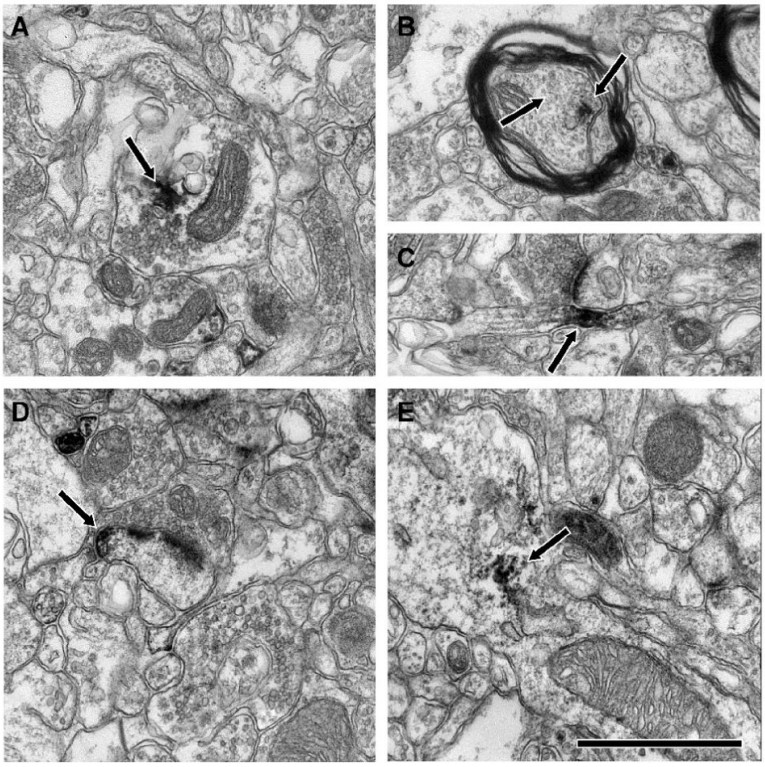

Figure 7.
Subcellular localization of MEF2A-ir in the LS/BNST. Immunoelectron microscopic analysis revealed MEF2A-ir in both axons and dendrites. Labeled axon terminals were particularly common (**A**, arrow). Preterminal myelinated (**B,** arrow) and unmyelinated (**C,** arrow) axons also contained MEF2A-ir. In addition, MEF2A-ir dendritic spines (**D**, arrow) and shafts (**E**, arrow) can be observed. Scale bar = 500 nm.

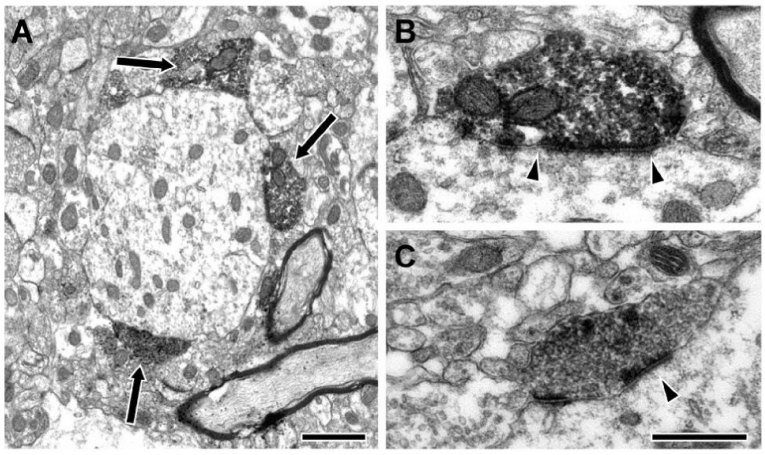

Figure 8.

MEF2A-ir in axon terminals in the LS/BNST. Intensely-labeled axon terminals (arrows) can be seen in apposition to large, proximal dendrites (**A**) or cell bodies (**B,C**). Symmetric synapses on the MEF2A-ir terminals are indicated by arrowheads. (Scale bar in A = 1 μm; scale bar in B and C = 500 nm).

TABLE 1

1Brain Areas Expressing MEF-2A-Immunoreactive Processes

Brain region		MEF2A-ir process density
Cortex	Prefrontal	-
	Motor	-
	Somatosensory	-
	Visual	-
	Pyriform	+
	Entorhinal	-
Basal Ganglia	Striatum, dorolateral	-
	Striatum, cental	-
	Striatum, medial	+
	Accumbens septal pole	+++
	Accumbens shell	++
	Accumbens core	+
	Striato-tubercle cell bridges	++
	Island of Calleja	-
	Olfactory tubercle	+
	Globus pallidus	-
	Ventral pallidum	++
	Subthalamic nucleus	-
Septal complex	Dorsolateral	+++
	Ventrolateral	+++
	Medial	+
	Bed nucleus stria, dorsal	++++
	Bed nucleus stria, ventral	++++
	Diagonal band of Broca	++
	Substantia innominata	-
Hippocampus	Stratum oriens	-
	Stratum pyramidale	-
Amygdala	Central, medial	++
	Central, lateral	+++
	Basal	-
	Basolateral	+
	Medial	+
	Posterior	-
	Intercalated	+
Thalamus	Anteroventral	-
	Anterodorsal	-
	Reuniens	++
	Paraventricular	+++
	Intermediodorsal	++
	Centrolateral	-
	Paracentral	-

Brain region		MEF2A-ir process density
	Parafascicular	+
	Ventroposterolateral/medial	-
	Medial geniculate	-
	Lateral geniculate	-
Hypothalamus	Paraventricular	++++
	Periventricular	++/+++
	Anterior hypothalamic area	+/++
	Medial preoptic	++
	Supraoptic	++/+++
	Dorsomedial	+++
	Ventromedial	++/+++
	Perifornical	+++
	Lateral	++/+++
	Arcuate	+++/++++
	Median eminence	+++/++++
Habenula	Medial	+
	Lateral	++
Midbrain	Ventral tegmental area	+
	Substanta nigra	-
	Retrorubral field	+/++
	Superior colliculus	-
	Inferior colliculus	-
	Red nucleus	-
	Deep mesencephalic nucleus	++
	Peduculotegmental nucleus	++
	Periaqueductal grey	+++
	Dorsal raphe	++/+++
	Median raphe	+/++
Pons	Locus coeruleus	++
	Lateraldorsal tegmental	++
	Dorsotegmental	+
	Parabrachial	-
	Reticular nuc, parvocellular	+++
	Superior and Inferior olivary	-

Plus signs (+) indicate relative densities of MEF2A-ir processes.

Minus signs (-) indicate the absence of MEF2A-ir fibers.