Protein expression pattern of human MIER1 alpha, a novel estrogen receptor binding protein

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Abstract

MIER1 is a transcriptional regulator that exists as several isoforms. Of particular interest is the MIER1 α isoform, which contains in its unique C-terminus an LXXLL motif for interaction with nuclear hormone receptors. Indeed, MIER1 α has been shown to interact with ER α and inhibit estrogen-stimulated growth of breast carcinoma cells. Moreover, the subcellular localization of MIER1 α changes dramatically, from nuclear to cytoplasmic, during progression to invasive breast carcinoma. While human MIER1 RNA and protein expression pattern data have been posted on several websites, none of these studies use probes or antibodies that distinguish between the α and β isoforms. We report here the first immunohistochemical study of the MIER1 α protein expression pattern in human tissues. Our analysis revealed intense staining of specific cell types within virtually every endocrine and reproductive tissue except for the thyroid gland. In particular, we detected intense staining of ovarian follicles and germinal epithelium, ductal epithelial cells of the breast, pancreatic islet cells, all areas of the anterior pituitary and all zones of the adrenal cortex; moderate staining of germ cells and Leydig cells within the testis, patches of chromaffin cells in the adrenal medulla and weak staining of the fibromuscular stroma within the prostate. Immunoreactivity was limited to the cytoplasm in all positive cells except for oocytes and germinal epithelial cells in which the nucleus was also stained and in ductal epithelial cells of the breast in which staining was exclusively nuclear. In general, non-endocrine tissues were negative, however a few exceptions were noted. These included hepatocytes, myocardial fibers and neurons in all regions of the brain examined, with the exception of the thalamus. Neuronal staining was restricted to the cell bodies and dendrites, as most axons were negative. These data suggest that human MIER1 α functions specifically in endocrine tissues and in a limited number of non-endocrine organs.

Keywords

mier1 α ; transcription factor; nuclear receptors; LXXLL motif; endocrine tissues; breast carcinoma; fibroblast growth factor

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INTRODUCTION

MIER1 was originally identified as a fibroblast growth factor (FGF)-activated gene in *Xenopus laevis* (Paterno et al. 1997). It has since been cloned and characterized in the mouse (Thorne et al. 2005) and in humans (Paterno et al. 1998; Paterno et al. 2002), displaying 95% overall similarity between these two species and 100% identity in most of the functional domains.

MIER1 is a single copy gene regulated by 2 promoters that give rise to multiple distinct mRNAs (Paterno et al. 2002). The resulting 4 MIER1 protein isoforms vary in their N- & C-terminal sequences, but share a common internal region that contains domains responsible for transcriptional regulation. Proteins with two functionally distinct N-termini have been identified: MIER1 and MIER1-3A. These two result from alternate promoter usage and splicing and differ by the presence of an N-terminal extension in the MIER1-3A isoform; this extension includes a *bona fide* nuclear export signal (NES) (Clements et al. 2012). The two variant C-termini, α and β , result from alternate inclusion of a facultative intron and differ both in size and in sequence (Paterno et al. 2002). The α C-terminus contains 23 amino acids and includes a classic LXXLL motif for interaction with nuclear hormone receptors, including ER α , while the β C-terminus contains 102 amino acids and possesses the only strong nuclear localization signal (NLS). Previous analysis by PCR revealed that the β isoform is more widely expressed and much more abundant than the α isoform (Paterno et al. 2002).

To date, most of the functional characterization has focused on MIER1 α and MIER1 β (Ding et al. 2003; Ding et al. 2004), providing evidence that both isoforms can function as transcriptional regulators, primarily through their effects on chromatin modifying enzymes: both recruit histone deacetylase 1 (HDAC1) and G9a methyltransferase (Wang et al. 2008) as well as inhibit CBP histone acetyltransferase activity (Blackmore et al. 2008). MIER1 can also repress its own promoter by an HDAC-independent mechanism that involves binding and displacement of Sp1 from its cognate sites in the promoter (Ding et al. 2004).

The MIER1 α isoform is of particular interest because of its potential role in breast cancer (McCarthy et al. 2008). MIER1 α has been shown to interact with ER α *in vitro* and regulated overexpression in breast carcinoma cells results in inhibition of anchorage-independent growth. Moreover, immunohistochemical analysis of its expression pattern in normal human breast and breast carcinoma samples revealed no difference in expression level but a dramatic shift in subcellular localization, from nuclear to cytoplasmic, during progression to invasive carcinoma. This pattern is distinct from that of many other genes (Jia et al. 2012) whose expression levels are increased breast carcinoma cells.

In spite of the potential link between loss of nuclear MIER1 α and the development of invasive breast carcinoma, the α -specific protein expression pattern in human tissues has not been determined. Reports of *mier1* RNA or protein expression data are available on websites, such as <http://www.ebi.ac.uk/gxa/gene/ENSG00000198160> or <http://www.proteinatlas.org/ENSG00000198160>, however all of these studies used probes or antibodies that target either the common region or β -specific sequence. Therefore, we

present here an immunohistochemical analysis of the α -specific expression pattern in human tissues/organs.

MATERIALS AND METHODS

Antibody and tissue sections

The MIER1 α antibody used for this study is a rabbit polyclonal antibody that had been prepared in our laboratory (Paterno et al. 2002) and is directed against a synthetic peptide containing alpha-specific sequence (amino acids 413-426). Its specificity for detecting MIER1 α protein in paraffin-embedded tissue sections has been described previously (McCarthy et al. 2008; Thorne et al. 2008). Briefly, the immunohistochemical specificity was tested by pre-incubation of the MIER1 α IgG for half an hour with 0.1 μ g of the alpha peptide used to generate the antibody or with 0.1 μ g of an unrelated peptide (control peptide), prior to application to the sections. Pre-absorption of anti-MIER1 α with alpha peptide resulted in complete loss of staining, while the control peptide had no effect. For the current study, we used purified anti-MIER1 α IgG and negative controls included sections stained with pre-immune IgG.

Paraffin sections of human adult tissues were purchased from Biochain Institute, Inc. (Medicorp Inc., Montreal, QC) and from US Biomax Inc. and tissue microarrays were from Biochain. According to the manufacturers, tissues were fixed for at least 48h in formalin, immediately after excision.

The tissues examined included testis, ovary, pancreas, adrenal gland, thyroid, prostate, breast, lung, liver, skeletal muscle, spleen, thymus, heart, kidney, salivary glands, small intestine, colon, brain. The latter included sections through the cerebrum, cerebellum, pituitary, medulla oblongata, pons, amygdala, thalamus, post- & pre- central gyrus. With the exception of the latter 6 regions of the brain, samples from more than one donor was examined (number of donors per tissue/organ are provided in Tables 1 and 2); 1–3 sections was analyzed for each. Donor ages ranged from 2–87 years old and a mixture of male and female samples were included, except for reproductive tissues.

Immunohistochemistry

Immunohistochemistry was performed using the Universal LSAB⁺-HRP kit (Dako, Denmark) and 1.25 μ g/ml of anti-MIER1 α IgG or pre-immune IgG, as described in (McCarthy et al. 2008) and applied universally to all samples. Briefly, sections were deparaffinized and rehydrated in a series of xylene and alcohol, according to the instructions provided by the manufacturer. Endogenous peroxidase activity was quenched by incubating the sections in 3.0% H₂O₂ for 10 min, as directed by Dako. Antigen retrieval was performed in 10mM sodium citrate, pH 6.0, in a 95°C water bath; optimum retrieval time was determined empirically to be 40 min for whole tissue sections and 30 min for TMAs. Slides were allowed to cool to room temperature, washed with phosphate-buffered saline (PBS) for 5 min, and non-specific binding sites blocked in the Dako LSAB blocking reagent for 20 min. Slides were incubated in a humidified chamber at 4°C overnight with primary antibody; as a control, each batch included slides stained with pre-immune IgG. Application of the

biotinylated secondary antibody followed by the streptavidin-HRP supplied with the Dako LSAB⁺-HRP kit was performed at room temperature for 15 min each, as directed by the manufacturer. MIER1 α was visualized using the HRP substrate 3, 3'-diaminobenzidine (DAB) and sections were counterstained with Mayer's hematoxylin, as described in (Wilkinson-Berka et al. 2003).

Evaluation of staining results

The staining of all sections was scored semi-quantitatively and independently assessed by two of the authors, PLM and LLG. The staining intensity was scored on a four-tiered system with – representing no staining, + weak staining, ++ moderate staining and +++ intense staining. Discrepancies were discussed, then a conclusive score assigned.

RESULTS and DISCUSSION

As observed in the mouse (Thorne et al. 2008), immunoreactivity for MIER1 α in adult humans was detected predominantly in tissues with endocrine function. The most intensely stained tissues/organs included ovary, testis, pancreas, adrenal gland and pituitary. A few non-endocrine tissues/cells consistently displayed intense immunoreactivity; these included neuronal cell bodies in several regions of the brain and cardiac muscle fibers. Additional, moderate staining was observed in the liver and endothelial lining of blood vessels, weak staining in the lungs and kidneys while the remaining tissues/organs were negative. With the exception of breast ductal epithelium, developing oocytes and the germinal epithelium of the ovary, no nuclear staining was observed; instead, MIER1 α was restricted to the cytoplasm of expressing cells. While MIER1 α 's reported activities are as a transcriptional repressor (Ding et al. 2003; Ding et al. 2004), its predominant cytoplasmic localization in most human tissues suggests that MIER1 α has additional function(s) in the cytoplasm. Such dual roles have been reported for other proteins such as ER α , which regulates transcription of target genes in the nucleus, but can also function to activate specific signal transduction pathways in the cytoplasm (reviewed in Simoncini et al. 2002; Simoncini et al. 2003). Alternatively, localization of MIER1 α in the cytoplasm might represent a control mechanism to regulate its effect on transcription. In light of the MIER1 α subcellular localization pattern described here, it will be important to investigate its role(s) in the cytoplasm.

A summary of MIER1 α immunoreactivity in endocrine and reproductive tissues is listed in Table 1 and in the remaining tissues/organs, Table 2.

MIER1 α expression in human reproductive tissues

In the ovary, immunoreactivity was restricted primarily to the follicles and germinal epithelium (Fig. 1, Table 1). Within the follicle, oocytes were intensely stained, while follicular cells showed moderate staining (Fig. 1c–f). Furthermore, both the nucleus and cytoplasm of the oocytes showed equal immunoreactivity, while only the cytoplasm of follicular cells was stained (white arrows in Fig. 1f). The level of immunoreactivity in the germinal epithelium was variable throughout the layer (Fig. 1g–h): a small proportion of cells were negative; the remainder displayed either moderate or intense staining and some germinal epithelial cells showed nuclear staining (Fig. 1h).

In the breast, immunoreactivity was detected primarily in ductal epithelial cells (Fig. 2) and was unique in that it was almost exclusively nuclear (arrowheads in Fig. 2b and arrows in Fig. 2f). However, not all ductal cells were stained (Fig 2b, d, f). In addition, the odd myoepithelial displayed weak immunoreactivity, while periductal fibroblasts and stroma were negative. This staining pattern is comparable to what we reported previously for human breast (McCarthy et al. 2008).

Germ cells at all stages of development in the seminiferous tubules of the testis showed weak to moderate staining, as did the Sertoli cells (Fig. 3b & d). Peritubular myoid cells (arrows in Fig. 3) were negative, as was the stroma. In the interstitium, Leydig cells (arrowheads in Fig. 3b & e) and blood vessels (bracket) displayed weak to moderate immunoreactivity. In the prostate gland, all epithelial cells lining the glands were negative, while smooth muscle cells located within the fibromuscular stroma showed weak staining (Fig. 3c). The transitional epithelium lining the prostatic urethra also showed weak immunoreactivity (arrow in Fig. 3f).

MIER1 α expression in human endocrine tissues

Of the endocrine tissues examined, only the thyroid showed no immunoreactivity (Fig. 4g–h); in the mouse, the follicular epithelium was also negative but parafollicular cells showed moderate staining (Thorne et al. 2008). Within the pancreas, the exocrine portion (acinar cells) was negative, the pancreatic ducts showed weak immunoreactivity and the islets were intensely stained (Fig. 4a–d). However, there were clusters of unstained cells within each islet (arrowhead in Fig. 4d); the identity of these cells is currently unknown.

In the adrenal gland, the capsule remained unstained however blood vessels within the capsule showed weak to moderate immunoreactivity (Fig. 5a–b). All three layers of the cortex showed intense immunoreactivity (Fig. 5a–c, e–g) however in regions containing little or no medulla, one could see a clear gradation of decreasing intensity from the outermost zona glomerulosa (ZG) to the innermost zona reticularis (ZR; Fig. 5b). Within the medulla of the adrenal gland, chromaffin cells were mostly negative, however patches of moderately stained cells were detected (Fig. 5c–d). This staining pattern is quite different from that previously reported for the mouse adrenal gland (Thorne et al. 2008). In the latter, the medulla displayed intense immunoreactivity while the X zone (equivalent to the ZR in humans) was negative; the mouse ZG was also intensely stained but the zona fascicularis (ZF) showed weak immunoreactivity.

MIER1 α expression in non-endocrine human tissues

We examined a variety of non-endocrine tissues/organs and, like that seen in the mouse (Thorne et al. 2008), most of these were showed little or no immunoreactivity (Table 2, Fig. 6). Such negative tissues included most smooth muscle, skeletal muscle (Fig. 6k), bone marrow, salivary glands (Fig. 6c), the cortex and medulla of the thymus (Fig. 6e–f), white and red pulp of the spleen (Fig. 6h), small intestine (Fig. 6d) and almost all of the colon (Fig. 6g). The one exception in the latter was Auerbach's plexus (myenteric plexus); some of the cells in this plexus showed moderate immunoreactivity (Fig. 7j–k). Auerbach's plexus is part of the enteric nervous system and is located between the longitudinal and circular layers

of the muscularis externa; it is the major nerve supply controlling motility of the gastrointestinal tract.

Weak staining was observed in some areas of the lung and kidney: a small number (<10%) of alveolar cells within the lung were positive and within the kidney, cortical labyrinths consisting of proximal and distal convoluted tubules were weakly positive, while medullary rays (loops of Henle) and glomeruli were negative (Fig. 6l).

Non-endocrine tissues showing significant immunoreactivity included the liver, heart and brain (Table 2, Fig. 6–7). Within the liver, all hepatocytes were moderately stained (Fig. 6b), while bile ducts and portal tracts (arrow in Fig. 6b), including the terminal portal vein located within, were negative. Endothelial cells lining the hepatic arterioles on the other hand showed weak immunoreactivity. In the heart, all myocardial fibers showed moderate to intense staining (Fig. 6i–j). Arteries were also stained (arrow in Fig. 6j) but not the surrounding connective tissue, fat (asterisk in Fig. 6j) or veins (arrowhead in Fig. 6j).

We examined several regions of the central and peripheral nervous system (Table 2 and Fig. 7); this included the spinal cord, peripheral nerves and several regions of the brain: the cerebrum, corpus callosum, cerebellum, medulla oblongata, pons, amygdala, thalamus, pituitary, pre- and post-central gyrus. The general trend that we observed was that the cell body and dendrites of most neurons showed intense immunoreactivity, while axons and glia were negative. In particular, we detected intense staining of pyramidal cells in the cortex (Fig. 7a–b), purkinje cells in the cerebellum and patches of neurons in other areas such as the pons (Fig. 7g–h) and medulla oblongata (Fig. 7f). The one exception to this trend was in the thalamus in which neither neurons nor glia were stained (Fig. 7c).

In summary, these results are generally consistent with the staining pattern reported for MIER1 α in the mouse (Thorne et al. 2008), with a few exceptions. Most notable was the difference in the staining pattern of the adrenal gland: the X zone of the mouse showed no immunoreactivity, while the equivalent ZR region of the human adrenal cortex was intensely stained. Also, in the mouse, the adrenal medulla was intensely stained while in humans, only patches of cells with moderate staining were detected. In addition, the subcellular localization of MIER1 α in the mouse was cytoplasmic in all tissues examined, while in a few human tissues (oocytes, mammary ductal epithelia and germinal epithelia), MIER1 α is localized in the nucleus. The predominant cytoplasmic localization of MIER1 α in both the mouse and humans suggests that it has additional non-genomic activities. Overall, this human MIER1 α staining pattern supports a role specifically in the endocrine system and in neuronal function.

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Figure 1.

MIER1 α expression in the ovary. Sections through the ovary stained with pre-immune (a, c, e, g) or immune (b, d, f, h) IgG, showing immunoreactivity in primary follicles (d & f) and germinal epithelium (arrows in h). Antibody staining is brown; counterstaining is blue. Both the nucleus (white arrow in f) and cytoplasm of oocytes (arrows in d & f) are stained, while in the follicular cells, only the cytoplasm is stained (arrowhead in f). Note that the surrounding stroma shows little or no staining (b, d, f, h). Some germinal epithelia cells show intense staining (black arrows in h), both in the cytoplasm and nucleus (green arrow) while other are not stained (arrowhead). Scale bar = 100 μ m in a–d and 50 μ m in e–h.

Figure 2. MIER1 α expression in breast tissue. Sections through normal human breast showing terminal duct lobular units (TDLU) (b, c, e) and ducts (a, d, f), stained with either pre-immune (a, c, e) or immune (b, d, f) IgG. Ductules (brackets in b) within the TDLU and ducts (d & f) show intense staining of the epithelial cells, which is almost exclusively nuclear (arrowheads in a and arrows in f). Periductal fibroblasts (red arrowhead in b) and stroma showed no immunoreactivity. Arrow in (b) indicates the nucleus of a myoepithelial cell. Scale bar = 50 μ m in a–d and 25 μ m in e–f.

Figure 3.

MIER1 α expression in testes and prostate gland. Pre-immune (a, d) and immune (b, e) staining of sections through human seminiferous tubules and prostate gland (c, f).

Arrowheads in (b) & (e) indicate moderately stained Leydig cells located in the interstitium and arrows indicate peritubular myoid cells. Note the even cytoplasmic staining of germ cells at all stages of spermatogenesis within the tubules. The bracket in (e) indicates a blood vessel. (c) shows the weakly stained smooth muscle within the stroma (fibromuscular stroma) surrounding the negatively stained prostatic epithelia cells lining the glands. (f) shows a section through the prostatic urethra; the thick epithelium is weakly stained. Scale bar = 100 μ m in a–c & f, 50 μ m in d–e.

Figure 4.

MIER1 α expression in the pancreas and thyroid gland. Pre-immune (a, c & e) and immune (b, d & f) staining of sections through the pancreas (a–d) and thyroid gland (e–f). Intensely stained islets (b and d) are shown surrounded by unstained acinar (exocrine) cells. Note that islet cells are either intensely stained or show no immunoreactivity (arrowhead in d). Arrow in c indicates an islet. Follicular epithelium (arrowheads) of the thyroid gland are shown in (f); note that no staining was detected in this gland. Scale bar = 250 μ m in a–b, 100 μ m in e–f, 50 μ m in c–d.

Figure 5.

MIER1 α expression in the adrenal gland. Sections through the adrenal gland in regions containing cortex (Cor) and medulla (Med) (a) or cortex only (b). The three regions of the cortex: zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) are indicated. The negatively stained capsule (Cap) is also indicated, as are blood vessels within the capsule (arrows). A higher magnification of the boxed in (a) is shown in (c) and further magnification of the boxed areas in (c) are shown in (d–g). M=medulla. Note that in the medulla, clusters of stained chromaffin cells are visible (d). Scale bar = 250 μ m in a–b, 100 μ m in c, 50 μ m in d–g.

Figure 6.

MIER1 α expression in the liver, digestive system, thymus, spleen, heart, skeletal muscle and kidney. Pre-immune (a, e, i) and immune (b, f, j) staining of the liver, thymus and heart, respectively. Immune staining (c–d, g–h & k–l) of the salivary gland, small intestine, colon, spleen, skeletal muscle and kidney, respectively. Arrow in (b) indicates a portal tract and the central lumen is the terminal portal vein. Note the weakly stained arterioles (arrowhead) within negatively stained portal tract. In the thymus (f), the medulla and cortex are indicated. In the heart (j), note that all cardiac muscle fibers are stained, while the connective tissue is not; the arrow indicates an artery, the arrowhead shows a vein and the asterisk indicates adipocytes. In the salivary gland (c), arrowheads indicate secretory units (acini) and asterisk shows adipocytes. The splenic nodules of the white pulp of the spleen (h) are indicated with white brackets; the arrow shows a weakly stained arteriole. In the kidney (l), medullary rays, consisting of loops of Henle, are indicated by a bracket and glomeruli by arrowheads; the cortical labyrinths (proximal & distal convoluted tubules) are indicated by a bar. Scale bar = 250 μ m in d, g–j & l, 100 μ m in a, c, e–f & k.

Figure 7. MIER1α expression in various region of the brain and in Auerbach's plexus. Staining of the cortex (a–b), thalamus (c), cerebellum (d–e), medulla oblongata (f), pons (g–h), anterior pituitary (h–i) and of Auerbach's plexus (j–k) in the colon. Neurons and glia are identified in various panels, as are pyramidal (Pyr) cells and purkinje (Pkj) cells. Note that neurons in the thalamus (c) show no immunoreactivity, unlike most other regions of the brain. In (d), the molecular (Mol) and granular (Gr) are indicated and in (e), D=dendrites. In Auerbach's plexus (j–k), intense staining of some of the cells was detected (arrowheads). Scale bar = 250μm in a, d, g; 100μm in c, f, i, j; and 50μm in b, e, h, k.

Table 1

MIER1α expression in endocrine and reproductive tissues

TISSUE/ORGAN	EXPRESSION	#DONORS ¹
Reproductive Organs		
<i>Breast</i>		8
Ductal epithelium	+++ N ²	
Myoepithelium	-/+	
Stroma	-	
Periductal fibroblasts	-	
<i>Ovary</i>		5
Germinal epithelium	++ N	
Stromal cells	-	
Follicular cells	++	
Oocytes	+++ N	
<i>Testis</i>		5
Interstitial:		
Leydig cells	+/++	
Stroma	-	
Seminiferous Tubules:		
Myoid cells	-	
Germ cells (all stages)	+/++	
Sertoli cells	+/++	
Endocrine Organs		
<i>Pancreas</i>		5
Acinar Cells	-	
Islets	-/++++	
Ducts	+	
<i>Adrenal</i>		5
Capsule:	-	

TISSUE/ORGAN	EXPRESSION	#DONORS ¹
Cortex:		
Zona glomerulosa	+++	
Zona fascicularis	+++	
Zona reticularis	+++	
Medulla:		
Chromaffin cells	-/++	
<i>Anterior pituitary</i>	++	3
<i>Thyroid</i>	-	3
<i>Prostate</i>		3
Glands: epithelial cells	-	
Smooth muscle	+	
Prostatic urethra-epithelium	+	

¹ Indicates the number of individual donor samples examined (1–3 sections each); donors ranged in age from 20–77 years (median age=36) and included a mixture of male and female samples (except for reproductive tissues).

² N indicates that nuclear staining was detected in that tissue; otherwise, only cytoplasmic staining was observed.

TABLE 2

MIER1 α expression in non-endocrine tissues

TISSUE/ORGAN	EXPRESSION ¹	#DONORS ²
Muscle		
Skeletal	-	3
Cardiac (myocardium)	++	4
Smooth	-	4
Kidney		3
Glomeruli	-	
Renal tubules	+	4
Lung	-/+	2
Digestive System		
<i>Salivary glands</i>	-	3
<i>Small Intestine</i>		3
Goblet cells	-	
Crypts	-	
Peyer's Patch	-	
<i>Colon</i>		3
Surface epithelium	-	
Columnar Epithelium	-	
Goblet cells	-	
Auerbach's plexus	++	
<i>Liver</i>		5
Hepatocytes	++	
Bile ducts	-	
Terminal portal vein	-	
Arterioles	+	
Terminal hepatic venule	-	
Blood vessels	++	3

TISSUE/ORGAN	EXPRESSION ¹	#DONORS ²
Bone Marrow	-	4
Lymphatic		
Thymus	-	4
Spleen (white & red pulp)	-	4
Nervous Tissue		
Spinal Cord	-	1
Peripheral Nerve	-	4
Brain		
Cerebrum:		5
Pyramidal Cells	+++	
Glia	-	
Corpus callosum	-	1
Cerebellum:		4
Stellate neurons (molecular layer)	+	
Glia	-	
Purkinje cells (body & dendrites)	+++	
Granule cells	+	
Medulla Oblongata:		1
Neurons (body & processes)	+++	
Glia	-	
Pons:		1
Neurons (body & processes)	+++	
Glia	-	
Amygdala:		1
Pyramidal cells	+	
Thalamus	-	1
Post-central gyrus	-	1
Pre-central gyrus	-	1

¹Note that all the staining pattern of all tissues was cytoplasmic only.

²Indicates the number of individual donor samples examined (1–3 sections each); donors ranged in age from 2–87 years (median age=41) and included a mixture of male and female samples.

³Staining intensity was evaluated in multiple tissue sections that contained identifiable vessels.