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Anti-Mullerian-hormone-dependent regulation of the brain serine-protease inhibitor neuroserpin

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Summary

The balance between tissue-type plasminogen activator (tPA) and one of its inhibitors, neuroserpin, has crucial roles in the central nervous system, including the control of neuronal migration, neuronal plasticity and neuronal death. In the present study, we demonstrate that the activation of the transforming growth factor- β (TGF β)-related BMPR-IB (also known as BMPRIB and Alk6)- and Smad5-dependent signalling pathways controls neuroserpin transcription. Accordingly, we demonstrate for the first time that anti-Mullerian hormone (AMH), a member of the TGF β family, promotes the expression of neuroserpin in cultured neurons but not in astrocytes. The relevance of these findings is confirmed by the presence of both AMH and AMH type-II receptor (AMHR-II) in brain tissues, and is supported by the observation of reduced levels of

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Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/121/20/3357/DC1>

Key words: Neuroserpin, Transcription, Anti-Mullerian hormone (AMH), Neuron, Brain, Serpin

Introduction

In the brain, tissue-type plasminogen activator (tPA, also known as PLAT) is implicated in many physiological functions (Benchenane et al., 2004; Samson and Medcalf, 2006) and in brain disorders such as epilepsy, multiple sclerosis and stroke (Tsirka et al., 1995; Tsirka et al., 1996; Wang et al., 1998; Nicole et al., 2001; Lu et al., 2002).

The activity of serine proteases depends on their equilibrium with serine-protease inhibitors, termed serpins, which generally act as pseudo-substrates for their target proteases. Protease nexin 1 (PN1, also known as SERPINE2) and type-1 plasminogen activator inhibitor (PAI-1, also known as SERPINE1) are the major inhibitors of thrombin- and plasminogen-activators, respectively. Neuroserpin (also known as SERPIN1) is another potent inhibitor of the proteolytic activity of tPA, and to a minor extent of urokinase-type plasminogen activator and plasmin, whereas it has no effect on thrombin (Osterwalder et al., 1998). Unlike other serpins, neuroserpin transiently complexes with tPA and thus acts as a competitive substrate. In contrast to the ubiquitously expressed PN1 and PAI-1, neuroserpin is almost exclusively expressed within the brain and spinal cord during both development and adulthood (Krueger et al., 1997).

tPA has been described to exert deleterious effects through its proteolytic activity following excitotoxic brain injuries (Benchenane et al., 2004), but these effects can be lessened by exogenous neuroserpin (Lebeurrier et al., 2005). Moreover, neuroserpin administration directly into the brain reduces ischemic lesions in rats following permanent middle-cerebral-artery occlusion (Yepes et al., 2000), whereas transgenic mice overexpressing neuroserpin exhibit smaller ischemic infarcts than wild-type littermates (Cinelli et al., 2001). The neuroprotective effect of neuroserpin following cerebral ischemia could also be explained by its ability to prevent the tPA-mediated blood-brain-barrier leakage (Zhang et al., 2002; Yepes et al., 2003).

Although the transcriptional regulation of tPA or PAI-1 is well described, that of neuroserpin is poorly characterized. In mouse hippocampal neurons, elevated extracellular concentrations of KCl induce neuroserpin gene transcription, whereas zif/268 (also known as Krox-24 and EGR1) has a repressive effect (Berger et al., 1999). Neuroserpin gene expression is also regulated at the level of RNA stability by the binding of HuD (also known as ELAVL4; an RNA-binding protein) to AU-rich sequences in its 3'-UTR or by a thyroid hormone (triiodothyronine, T₃) in PC12 cells and in the rat brain (Cuadrado et al., 2002; Navarro-Yubero et al., 2004). More recently,

the neuroserpin promoter was reported to be placed head-to-head with the programmed-cell-death-related gene *PDCD10*, leading to a co-regulation of both genes (Chen et al., 2007).

Anti-Mullerian hormone (AMH), also named Mullerian inhibiting substance (MIS), belongs to the transforming growth factor- β (TGF β) superfamily (Massague, 1998), and is known to transduce its signals through serine/threonine type-II and type-I receptors. To date, only the AMH type-II receptor (AMHR-II, also known as AMHR2) has been identified (di Clemente et al., 2003); the type-I receptor (AMHR-I) remains to be clearly identified. Candidates for AMHR-I are the bone morphogenetic protein (BMP) type-I receptors activin receptor-like kinase 2 (Alk2, also known as ACVR1), Alk3 (also known as BMPR1A and BMPR-IA) and Alk6 (also known as BMPR1B and BMPR-IB) (Josso et al., 2001; di Clemente et al., 2003).

The aim of the present study was to reveal some elements of the transcriptional regulation of neuroserpin in the brain. By using a set of luciferase reporter genes for neuroserpin, we unexpectedly identified AMH as a candidate regulator of neuroserpin expression. Accordingly, by using primary cultures of cortical neurons and AMHR-II-deficient mice, we revealed that AMH should now be considered as a new regulator of neuroserpin expression and as a neuroprotective factor.

Results

A Smad-responsive element controls the transcriptional regulation of neuroserpin

The mink lung epithelial cell line (Mv1Lu) was transiently transfected with the firefly luciferase reporter gene placed under the control of various domains of the mouse neuroserpin promoter, allowing us to identify several regulatory sequences within the neuroserpin promoter (see Fig. 1 for details of the different constructs). Luciferase activity (Luc) increased when the region -1135 to -511 [LucC=454 \pm 87.40% versus LucD=1228.11 \pm 166.41%; $P<0.01$], -511 to -346 (LucD=1228.11 \pm 166.41% versus LucE=3008.08 \pm 598.80%; $P<0.01$) or -78 to -24 (LucG=211.71 \pm 38.11% versus Luc24=1687.69 \pm 335.11%; $P<0.01$) of the neuroserpin promoter was absent. This finding suggests the presence of silencer elements in each of these domains. By contrast, luciferase activity decreased when the region -346 to -125 (LucE=3008.08 \pm 598.80% versus LucF=1215 \pm 304.19%; $P<0.05$) or the regions -125 to -78 (LucF=1215 \pm 304.19% versus LucG=211.71 \pm 38.11%; $P<0.01$) was absent. This result indicates the presence of enhancer elements in these two regions of the neuroserpin promoter.

In order to establish a link between the domains identified above and known responsive elements, a sequence analysis of the neuroserpin promoter was performed using the Genomatix software. Among the responsive elements identified, we noted that the E construct (-346 to +72) contains a positive responsive element between the positions -317 and -310, which matches the sequence GTCTAGCC, previously described as a typical Smad-binding element (SBE) that is crucial for TGF β -family-mediated cell responses (Dennler et al., 1998).

On the basis of these observations, Mv1Lu cells were transiently co-transfected with the cDNAs encoding for mutated forms of the type-I receptors for members of the TGF β family [Alk proteins carrying mutations that lead to the auto-activation of the receptor; see Nakao et al., 1997 (Nakao et al., 1997)] and the A (full-length promoter) or E (-346 to +72) constructs. BMPR-IA, BMPR-IB and ActR-I (also known as ACVR1 and Alk2) are BMP type-I receptors, whereas TGF β and activin mediate their signal through

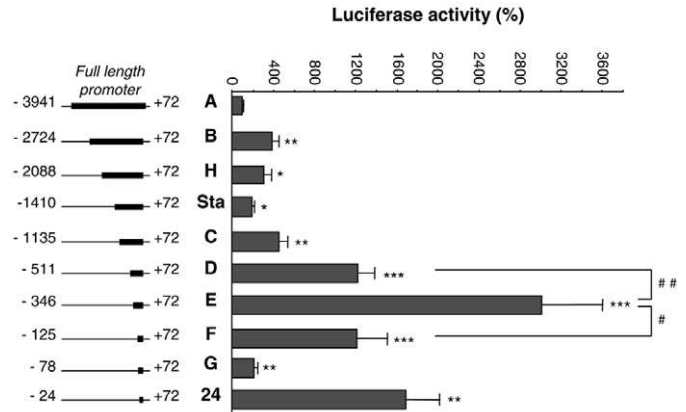


Fig. 1. Analysis of the putative mouse neuroserpin promoter. Regions of the putative mouse neuroserpin promoter were subcloned into pGL3-Basic vector, upstream of the firefly luciferase gene. Mv1Lu (mink lung epithelial) cells were transiently transfected with the pRL-TK control vector and pGL3-Basic vector containing the different regions of the mouse neuroserpin promoter. Firefly and *Renilla* luciferase activities were measured as described in the Materials and Methods. After normalization to *Renilla* luciferase activity, values were expressed as a percentage of the activity of construct A (valued as 100%) (mean \pm s.d.; $n=8$). Asterisks indicate a significant difference from the A construct: * $P<0.05$, ** $P<0.01$, *** $P<0.001$; hash marks indicate a significant difference from the E construct: # $P<0.05$, ## $P<0.01$; Kruskal-Wallis test followed by a Mann-Whitney post-hoc test.

T β R-I (also known as TGFBR1 and Alk5) and ActR-IB (also known as ACVR1B and Alk4) (Massague, 1998), respectively. AMH mediates its signal through Alk2, Alk3 or Alk6 (di Clemente et al., 2003) (Fig. 2D). The overexpression of Alk6 increased the transcriptional activity of the E construct (LucE=99.92 \pm 1.86% versus LucE/Alk6=809.67 \pm 86.47%; $n=12$; $P<0.001$) (Fig. 2A); Alk3 and Alk4 enhanced the activity of the E construct to a weaker extent. The other Alk proteins had no effect (Fig. 2A). Similarly, Alk6 promoted the transcriptional activity of the full-length neuroserpin promoter (LucA=99.96 \pm 3.49% versus LucA/Alk6=701.83 \pm 153.90%; $n=11$; $P<0.001$) (Fig. 2B). The other Alk proteins had no effect, apart from a slight transcriptional activation of the neuroserpin promoter with Alk3 and Alk4 (Fig. 2B). These results suggest that the transcriptional activity of the neuroserpin promoter could be potentially enhanced both by BMPs and AMH, and to a lesser extent by TGF β and activin.

All receptors for the TGF β family are known to mainly transduce their signals through the activation of Smad-dependent signalling pathways. Thus, we transiently co-transfected Mv1Lu cells with expression vectors encoding for different members of the Smad family and the E construct. Smad2 and Smad3 are specific mediators of the TGF β and activin pathways, whereas Smad1, Smad5 and Smad8 are involved in BMP signalling (Massague, 1998) and AMH signalling (di Clemente et al., 2003) (Fig. 2D). Among the different Smad proteins that were tested, only Smad5 significantly increased the transcriptional activity of the E construct (LucE=100.40 \pm 1.83% versus LucE/Smad5=205.98 \pm 21.52%; $n=11$; $P<0.001$) (Fig. 2C). These data highlight BMPs and/or AMH as potential regulators of the transcription of the neuroserpin gene.

AMH controls the cerebral expression of neuroserpin both in vitro and in vivo

Based on the foregoing results, the influence of recombinant BMPs and/or AMH on neuroserpin gene expression was investigated by

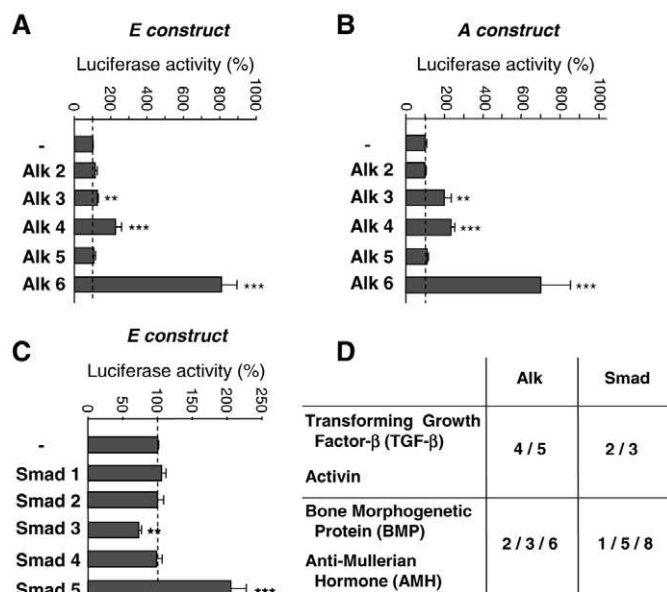
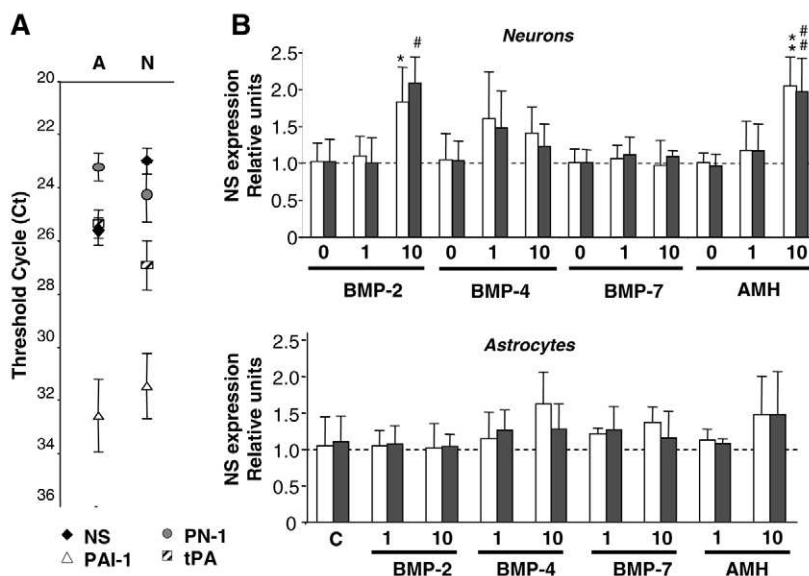


Fig. 2. AMH promotes the transcription of neuroserpin. (A,B,C) Mv1Lu cells were transiently transfected with the pRL-TK control vector and pGL3-Basic vector containing the E (-346 to +72) or A (-3941 to +72) constructs. At the same time, cells were co-transfected with pcDNA3 containing the cDNA encoding for either auto-activated versions of type-I receptors for members of the TGF β family (Alk proteins, A,B) ($n=11-12$) or proteins of the Smad family (C) ($n=8$). Luciferase activities were measured as described in Materials and Methods. Values are indicated as a percentage of the activity in controls (-; E or A reporter vector co-transfected with the empty pcDNA3 vector). Bars represent mean values \pm s.d. ** $P<0.01$, *** $P<0.001$; Kruskal-Wallis test followed by Mann-Whitney posthoc test. (D) Summary of the Alk and Smad proteins involved in intracellular signalling pathways of the TGF β family members TGF β , BMP and AMH.

quantitative reverse-transcriptase (RT)-PCR both in murine cultured cortical neurons and astrocytes. Under basal conditions, both neurons and astrocytes displayed mRNA for the serine protease tPA and its endogenous inhibitors neuroserpin, PN1 and, to a lesser extent, PAI-1 (Fig. 3A).

Primary cultures were then treated with recombinant BMPs (BMP2, BMP4 and BMP7), at a dose of 1 or 10 ng/ml, over 24 hours. Whereas BMP4 and BMP7 had no significant effect in both cell types (Fig. 3B; Fig. 4), BMP2 selectively upregulated neuroserpin transcription (Fig. 3B) but not neuroserpin translation (Fig. 4) in neurons, with no effect in astrocytes.

Fig. 3. Regulation of neuroserpin expression in vitro. (A) mRNA levels of neuroserpin (NS), tPA, PAI-1 and protease nexin 1 (PN1) in cultured neurons (N; $n=3$) and astrocytes (A; $n=3$). (B) Evaluation of the mRNA levels for neuroserpin in cultured neurons ($n=5$) and astrocytes ($n=3$) treated over 24 hours with either recombinant BMP2, BMP4, BMP7 or AMH at a concentration of 1 or 10 ng/ml. Relative levels of mRNA expression were measured by quantitative PCR as described in the Materials and Methods. C, control. Results were computed by calculating both the $2^{-\Delta Ct}$ (white bars) and the $2^{-\Delta\Delta Ct}$ (grey bars). Bars represent mean values \pm s.d. ($2^{-\Delta Ct}$: * $P<0.05$, ** $P<0.01$; $2^{-\Delta\Delta Ct}$: # $P<0.05$, ## $P<0.01$; Kruskal-Wallis test followed by Mann-Whitney post-hoc test).



Primary cultures were then exposed to recombinant AMH (1 or 10 ng/ml) for 24 hours in serum-free medium. In cultured cortical neurons, AMH (10 ng/ml) doubled the transcription of the neuroserpin gene ($n=5$; $P<0.01$) (Fig. 3B) whereas, in astrocytes, there was a tendency towards activation, but that did not reach statistical significance ($n=3$) (Fig. 3B). AMH also failed to promote the expression of mRNA encoding for the programmed-cell-death-related gene PDCD10, a gene that was recently demonstrated to be linked by an asymmetric bidirectional promoter to neuroserpin (Chen et al., 2007) (data not shown). In agreement with the PCR analyses, immunoblots that were performed on cell monolayers revealed that, in cultured cortical neurons, AMH increased neuroserpin protein levels by 50% after 24 hours (Fig. 4) ($n=11$; $P<0.01$).

The expression of neuroserpin was also investigated in several brain structures (cortex, striatum, hippocampus) of AMHR-II-deficient mice (heterozygous and homozygous deficient animals) and wild-type littermates (Fig. 5). Quantitative RT-PCR revealed that AMHR-II deficiency led to reduced cerebral levels of mRNA encoding for neuroserpin in these structures (i.e. ~35% in the cortex; $n=6-12$; $P<0.05$) (Fig. 5). Because AMH is known to control sexual maturation (Josso et al., 1998; Josso and di Clemente, 1999), the effect of endogenous AMH on the control of brain neuroserpin expression was investigated in both males and females. Gender did not affect the expression of neuroserpin in the adult brain (see supplementary material Fig. S1).

Altogether, these data demonstrate that AMH is a regulator of the neuronal expression of neuroserpin both in vitro and in vivo.

AMHR-II receptors are expressed in the developing and adult brain

To determine whether AMHR-II is expressed in the brain, immunohistochemistry with previously characterized antibodies (Gouédard et al., 2000) was performed on mouse embryos (E16, corresponding to the developmental stage from which cultures of cortical neurons were obtained) and adult mice. Immunostaining for BMPR-II was performed in parallel in order to check that the antibody used against AMHR-II did not crossreact with BMPR-II. Antibodies revealed different specific antigens both in the brain (see

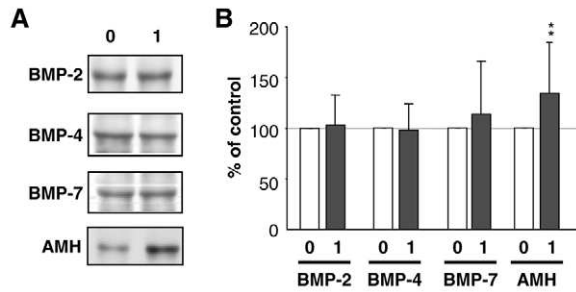


Fig. 4. AMH promotes the expression of the protein neuroserpin in cultured neurons. (A) Neurons were treated during 24 hours with either recombinant BMP2 ($n=9$), BMP4 ($n=6$), BMP7 ($n=3$) or AMH ($n=7$) at the concentration of 1 ng/ml. Immunoblots were revealed with an antibody raised against mouse neuroserpin [antibody G64 (Lebeurrier et al., 2005)]. (B) Densitometries of immunoblots performed with ImageJ software. Bars represent mean values \pm s.d. $**P<0.01$; Kruskal-Wallis test followed by Mann-Whitney post-hoc test.

supplementary material Fig. S2B,D,F) and the testes (see supplementary material Fig. S2A,C,E). Thus, we first studied the expression pattern of AMHR-II in the mouse telencephalic wall at E16. We observed that the AMHR-II immunostaining (TRITC) was associated with the developing ventricles (Fig. 6A-D), especially the ventricular cell layer and the surrounding subventricular zone (Fig. 6C,D). In the adult mouse (C57Bl6/J and Swiss) brain, immunostaining for AMHR-II (TRITC) was mainly associated with brain capillaries (Fig. 7A-D). AMHR-II-positive staining was also localized in the cortical (Fig. 7A-D) and hippocampal (Fig. 7E,F) parenchyma, with an intense staining around large vessels of the stratum lacunosum molecularis (SLM) (Fig. 7E), in which the density of glial cells is known to be high (Shimada et al., 1991). Fig. 7F illustrates AMHR-II-positive staining in glial cells (white arrows). In addition, AMHR-II immunoreactivity was also noted along nerve fibres (as illustrated by neurofilament-positive staining, Fig. 7D) (NF: FITC) in the cortex (Fig. 7C,D), but also in the cerebellum (see supplementary material Fig. S3A) and the corpus callosum (see supplementary material Fig. S3B), suggesting the presence of AMH-RII immunoreactivity in adult neuronal cells.

The expression pattern of AMH and AMHR-II in the brain was further characterized by quantitative RT-PCR that was performed on RNAs harvested from either primary cultures [neurons at 2 or 14 days in vitro (DIV), astrocytes and brain-derived endothelial cells] or nervous-system structures (cortex and spinal cord from neonatal or adult mice; Table 1). Positive expression of T β R-II (also known as TGFBR2; one of the two receptors known to transduce TGF β 1 signalling) and of TGF β 1 were investigated in parallel. The findings show that, similar to T β R-II ($Ct_{\text{Neuron } 14\text{DIV}}=27.0\pm 0.4$; Ct denotes threshold cycle), AMHR-II is expressed in neurons ($Ct_{\text{Neuron } 14\text{DIV}}=29.3\pm 1.0$). Cultured astrocytes and, to a lesser extent, cultured brain endothelial cells (Table 1) also exhibit AMHR-II. mRNA encoding for AMHR-II were also detected in the neonatal (P7) and adult brain cortex. As previously described (Wang et al., 2005), AMHR-II is also expressed in the spinal cord from neonatal and adult mice. Although the levels of mRNA encoding for AMHR-II were significantly different in neonatal mice with respect to gender, no such effect of sex was observed in adult mice (Table 1).

The expression of AMH mRNA was also investigated in cultured cells and tissues. Cultured neurons, astrocytes and brain endothelial cells displayed mRNA encoding for AMH as they did for TGF β 1

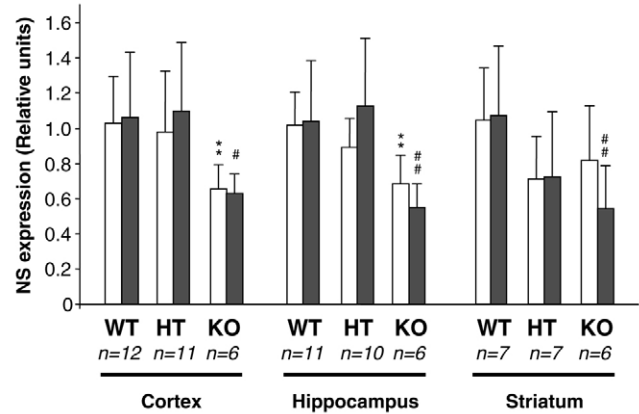


Fig. 5. Expression of neuroserpin in AMHR-II-deficient mice. Evaluation of the mRNA levels of neuroserpin (NS) in the cerebral cortex, hippocampus and striatum of C57Bl6/J mice. Mice strains are wild type (WT; $n=7-12$), or heterozygote (HT; $n=7-11$) or homozygote (KO; $n=6$) AMHR-II deficient. Results were computed by calculating both the $2^{-\Delta Ct}$ (open bars) and the $2^{-\Delta\Delta Ct}$ (filled bars). Symbols indicate significant difference from wild-type mice ($2^{-\Delta Ct}$: $**P<0.01$; $2^{-\Delta\Delta Ct}$: $##P<0.01$); Kruskal-Wallis test followed by Mann-Whitney post-hoc test.

(in neurons at 14 DIV: $Ct_{\text{AMH}}=28.7\pm 0.8$ versus $Ct_{\text{TGF}\beta 1}=27.3\pm 0.3$). AMH was also expressed in cortices from neonatal and adult mice. In the spinal cord, AMH expression was lower in adult mice than in neonatal mice. Interestingly, both neonatal and adult female mice displayed a lower expression of AMH in the cerebral cortex than did their male counterparts. This gender effect was also true for neuroserpin mRNA in neonatal mice (Table 1).

AMH protects neurons against NMDA-mediated excitotoxicity
Because neuroserpin was previously demonstrated to protect neurons from NMDA-mediated death both in vitro and in vivo (Lebeurrier et al., 2005), we hypothesized that, if AMH actually promotes neuroserpin expression, it should also be able to exert a neuroprotective activity. First, primary cultures of cortical neurons that were subjected to NMDA-induced excitotoxic neuronal death were thus treated with increasing doses of recombinant AMH (1-10 ng/ml) (Fig. 8A). Although NMDA alone induced around 50% neuronal death, co-application of recombinant AMH at 10 ng/ml led to a significant reduction of NMDA-induced neuronal death (65% reduction), as previously reported for neuroserpin. Similarly, in a model of excitotoxic neuronal death in mice, induced following intrastriatal injection of NMDA (10 nmol) (Fig. 8B), co-injection of recombinant AMH (0.45 μ g) led to a reduction of around 35% of lesion volume ($P<0.01$).

Discussion

Although the cerebral distribution of the neuroprotective serpin neuroserpin is well characterized, its transcriptional regulation is poorly documented. Neuroserpin is expressed in the developing and adult nervous system (Krueger et al., 1997). In situ hybridization has previously revealed a neuronal expression during the late stages of neurogenesis and, in the adult brain, in regions that exhibit synaptic plasticity (Osterwalder et al., 1996). During embryonic development, neuroserpin is expressed in several central nervous system (CNS) regions. In the cerebellum, the granule cells and a subgroup of Purkinje cells display neuroserpin during postnatal development (Krueger et al., 1997). In the adult CNS, neuroserpin

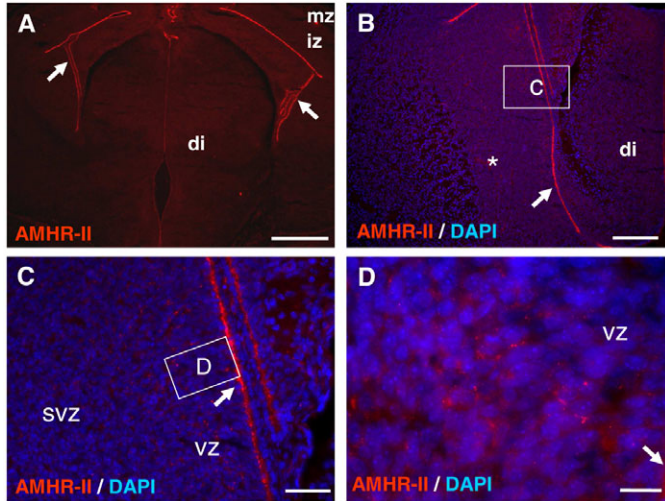


Fig. 6. Distribution of AMHR-II immunoreactivity in the developing forebrain of E16 mouse embryos (coronal sections). (A-D) AMHR-II immunoreactivity (TRITC) is detected in the neuroepithelium of the ventricular zone (arrows). AMHR-II immunoreactivity is also detected in the developing parenchyma (* in B). Higher magnifications of the boxed region in B and C (C and D, respectively) show that the AMHR-II immunostaining is mainly confined to the ventricular zone (VZ) and the subventricular zone (SVZ). (B-D) Nuclei are counterstained with DAPI (blue). di, diencephalon; iz, intermediate zone; mz, marginal zone. Scale bars: 500 μ m (A); 200 μ m (B); 50 μ m (C); 20 μ m (D).

is expressed in the neocortex, hippocampus, olfactory bulb and amygdala (Hastings et al., 1997). Expression of neuroserpin has also been reported in the spinal cord (Osterwalder et al., 1996; Hastings et al., 1997; Krueger et al., 1997). Our present data are in agreement with these previous reports. Regarding its transcriptional regulation, the level of neuroserpin mRNA is increased in cultured hippocampal neurons upon depolarization (Berger et al., 1999). Using luciferase reporter constructs that contained segments of the promoter region of the neuroserpin gene, Berger and collaborators identified a 200-bp segment, near the transcription initiation site, that was responsible for both the neuron-specific expression and the enhanced transcription following depolarization. Similarly, neuroserpin mRNA and protein expression are induced by the

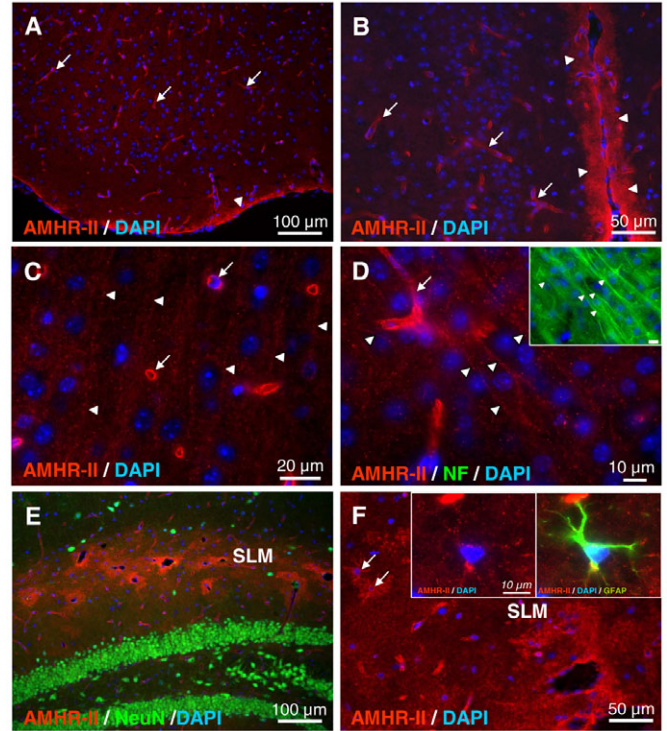


Fig. 7. AMHR-II immunostaining in adult mouse brain. In the cortex, immunostaining for AMHR-II (TRITC) is predominantly associated with capillaries (arrows in A-D) and the external zone of the cortical layer (arrowheads in A and B), with a weaker immunostaining in the parenchyma. In the cortex, AMHR-II immunostaining is associated with cortical nerve fibres (arrowheads in C and D), which are positive for the 160-kDa neurofilament (insert in D). In the hippocampus (E), immunostaining for AMHR-II (TRITC) is observed in the parenchyma surrounding the large vessels of the stratum lacunosum molecularis (SLM). At higher magnification (F), the perivascular AMHR-II immunoreactivity is associated with glial cells (arrows) and, more precisely, astrocytes, which are characterized by GFAP immunoreactivity (inserts in F). The nuclei are counterstained with DAPI (blue; A-F).

thyroid hormone in rat PC12 cells (Cuadrado et al., 2002; Navarro-Yubero et al., 2004). Although there is no direct effect on the transcription rate, the neuroserpin mRNA half-life is increased

Table 1. Expression of AMHR-II, AMH and neuroserpin in the central nervous system

			AMH-RII	AMH	T β R-II	TGF β	Neuroserpin
Cultures	Neurons	2 DIV	28.9 \pm 0.81	27.6 \pm 0.43	26.77 \pm 0.34	25.79 \pm 0.52	–
		14 DIV	29.35 \pm 1.01	28.72 \pm 0.77	27.04 \pm 0.37	27.3 \pm 0.3	23 \pm 0.49
	Astrocytes	–	29.08 \pm 0.34	30.51 \pm 0.57	–	–	25.64 \pm 0.54
Microvessels	Endothelial cells	From P10	33.08 \pm 1.13	29.78 \pm 0.45	–	–	–
		From adult	33.38 \pm 0.79	29.86 \pm 0.34	–	–	–
			From P10	31.11 \pm 1.24	29.88 \pm 0.13	–	–
Neonatal mice (P7)	Cortex	From adult	30.82 \pm 1.64	29.16 \pm 0.78	–	–	26.23 \pm 0.48
		Male	29.64 \pm 0.62	31.11 \pm 0.45	–	–	22.48 \pm 0.46
		Female	31.29 \pm 1.2	33.12 \pm 1.55	–	–	24.52 \pm 1.67
	Spinal cord	Male	30.91 \pm 0.38	31.34 \pm 0.66	–	–	22.63 \pm 0.63
Female		31.75 \pm 0.58	31.87 \pm 0.71	–	–	24.11 \pm 0.95	
Adult mice	Cortex	Male	29.89 \pm 0.27	31.27 \pm 0.48	27.94 \pm 0.25	26.6 \pm 0.37	22.13 \pm 0.51
		Female	29.69 \pm 0.37	32.27 \pm 0.58	27.66 \pm 0.29	26.06 \pm 0.13	21.63 \pm 0.45
	Spinal cord	Male	28.09 \pm 1.12	35.06 \pm 2.34	–	–	26.39 \pm 0.66
		Female	28.32 \pm 1.03	34.17 \pm 2.09	–	–	27.59 \pm 1.29

Evaluation of mRNA levels of AMHR-II, AMH, T β R-II, TGF β and neuroserpin in either cultured cells such as neurons (2 or 14 DIV, $n=3$), astrocytes and endothelial cells (from P10 or adult Swiss mice, $n=3-4$), or in nervous tissue such as cerebral cortex and spinal cord (from P7 or adult Swiss mice; males or females; $n=6$ per group), and in microvessels (from P10 or adult Swiss mice; $n=3$). The values (\pm s.d.) correspond to the mean threshold cycle (C_t).

following binding of a cytoplasmic protein, the RNA-binding protein HuD, to the neuroserpin 3'-UTR, leading to an increased accumulation of neuroserpin mRNA (Cuadrado et al., 2002; Navarro-Yubero et al., 2004). In the present study, we provide evidence that a 5' regulatory element (-317 to -310) of the rat neuroserpin promoter, characterized as a Smad-binding element (SBE), positively controls the transcription of neuroserpin. Indeed, transient transfection of either auto-activated Alk6 or Smad5 in Mv1Lu cells leads to an increased transcriptional response of the neuroserpin promoter. In our hands, although our data are in agreement with those published by Berger et al. (Berger et al., 1999) and demonstrate the presence of an enhancer region of 200 bp near the transcription initiation site of the neuroserpin promoter, they differ from an earlier study (Chen et al., 2007) that reported a repressive regulatory element in position 176-473, which was absent in our system. These latter discrepancies could be due to species differences (murine versus human) and/or to the type of cell line used for reporter-gene experiments. Accordingly, we demonstrate that AMH, a member of the TGF β superfamily, regulates the expression of neuroserpin in neurons but not in astrocytes, supporting previous reports suggesting that neurons are the major source of neuroserpin in the CNS (Krueger et al., 1997). Although astrocytes display AMHR-II, neuroserpin transcription was weakly induced by AMH, but not to levels reaching statistical significance. Among other explanations, one can hypothesize that additional elements in the transduction of AMH signal (such as the associated AMHR-I) might lack in astrocytes, and/or that these cells alternatively express inhibitory factors (repressive transcription factors, inhibitory Smads etc.). Our *in vivo* data obtained from AMHR-II-deficient-mice confirm the relevance of the transcriptional regulation of brain neuroserpin by AMH.

BMPs and AMH are members of the TGF β superfamily, which includes over 25 members in mammals. Several of these members have been reported to be expressed in the CNS, such as TGF β (Vivien and Ali, 2006) and BMPs (Ebendal et al., 1998). TGF β and BMPs mediate their signal via different hetero-oligomeric complexes of type-I and type-II serine/threonine kinase receptors. The adult brain exhibits most of these receptors, including high levels of BMP receptor type II, TGF β receptors type I and II, and activin receptors type I and II (Ebendal et al., 1998; Vivien and Ali, 2006). These observations suggest that TGF β s (TGF β 1, TGF β 2 and TGF β 3), osteogenic protein 1 (also known as OP-1 and BMP7), BMP2 and BMP4 exert a specific neuronal function. AMHR-II has been identified and cloned (di Clemente et al., 2003); however, no clear AMHR-I has been reported so far (Josso et al., 2001; di Clemente et al., 2003). Intracellular signalling mediating responsiveness to AMH involves BMP-like signalling pathways such as recruitment of the transcription factors Smad1, Smad5 and Smad8 (Josso et al., 2001; di Clemente et al., 2003). Although AMH signalling has been reported to play a crucial role during sex differentiation and gonadal functions (Josso et al., 1998; Josso and di Clemente, 1999), a recent report suggests that AMH-dependent signalling could control the survival of motor neurons (Wang et al., 2005). Accordingly, the presence of AMHR-II was demonstrated in motor neurons (Wang et al., 2005). We now provide evidence that cerebral neurons also exhibit AMHR-II (mRNA and protein) both *in vitro* and *in vivo*. Interestingly, AMHR-II was also detected in brain microvessels and to a lesser extent in cultured endothelial cells. Although the ligand of this receptor, AMH, was initially reported to be expressed by both Sertoli cells in the testes (Josso, 1973) and in granulosa cells in ovaries (Vigier et al., 1984), it was

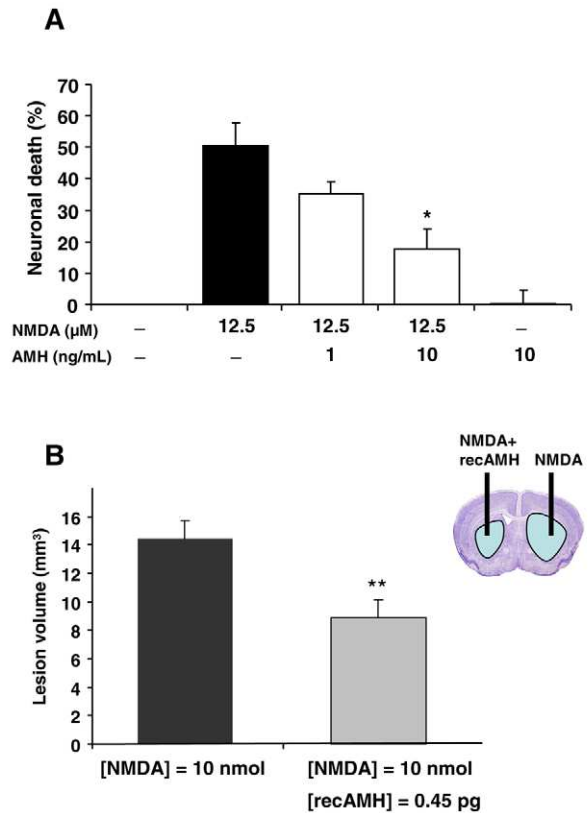


Fig. 8. Neuroprotective effect of AMH against NMDA-induced excitotoxicity. (A) Pure cultures of neurons (14 DIV) were exposed for 24 hours to NMDA (12.5 μ M) without (black bars) or with (white bars) increasing concentrations of AMH (1 or 10 ng/ml) and neuronal death was measured as described in the Materials and Methods ($n=4$). Asterisks indicate a significant difference from NMDA alone by one way Kruskal-Wallis test followed by Mann-Whitney post-hoc test ($*P<0.05$). (B) Effects of intra-striatal injection of recombinant AMH (0.45 pg) on the extent of neuronal death induced by the striatal administration of NMDA (10 nmol) in mice ($n=9$). Bars represent mean values \pm s.e.m. $**P<0.01$; Mann-Whitney test. Diagram on the right illustrates on a brain section the typical lesion size obtained by the striatal injection of NMDA alone versus NMDA + AMH.

also recently described in the spinal cord (Wang et al., 2005). Here, we thus extend these works by showing that the mRNA encoding for AMH is also expressed in the brain parenchyma.

One of the main roles of neuroserpin in the CNS is the control of the proteolytic activity of the serine protease tPA. Serine proteases are a superfamily of proteins in which most members were initially described for their functions in relation to coagulation and thrombolysis (Lijnen and Collen, 1997). In addition to their presence in the blood, several serine proteases, including tPA, have been identified in the CNS. Within the brain parenchyma, tPA is synthesized by neurons and glial cells (Buisson et al., 1998; Rogove and Tsrka, 1998). tPA is now considered as a positive neuromodulator of the glutamatergic signalling involved in learning and memory processes (Qian et al., 1993; Huang et al., 1996; Fernandez-Monreal et al., 2004a; Samson and Medcalf, 2006) and in some neurodegenerative diseases (Melchor et al., 2003). For example, it was shown that tPA can interact with the NMDA receptor and thus potentiates excitotoxic neuronal death (Nicole et al., 2001; Fernandez-Monreal et al., 2004b). Accordingly, tPA inhibitors, such as PAI-1 and neuroserpin, are able to counteract this deleterious effect of tPA in the brain. For example, studies have revealed that

PAI-1 can prevent the harmful effect of tPA against excitotoxic or ischemic brain injuries (Nagai et al., 1999; Nicole et al., 2001; Benchenane et al., 2005). Similarly, neuroserpin was shown to be neuroprotective in a rat model of stroke (Yepes et al., 2000) and to promote survival of motor and cortical neurons (Simonin et al., 2006; Lebeurrier et al., 2005). In agreement with these observations and with the recent demonstration that AMH-dependent signalling could also control the survival of motor neurons (Wang et al., 2005), we evidence here that AMH protects neurons against NMDA-mediated excitotoxicity both in vitro and in vivo. Although we cannot rule out the possibility of other mechanisms, all our data strongly suggest that AMH-induced expression of neuroserpin in neurons could sustain a neuroprotective pathway against NMDA-receptor-mediated excitotoxicity.

In conclusion, we evidence that AMH should now be considered as a new cytokine or hormone in the brain, with the serine-protease inhibitor neuroserpin as one of its target genes, which could confer neuroprotection against excitotoxicity.

Materials and Methods

Reporter-gene assay

The Dual-luciferase Reporter Assay System (Promega, France) refers to the simultaneous expression and measurement of two individual reporter enzymes (firefly luciferase and *Renilla* luciferase) within a single system. The pRL-TK vector containing the herpes simplex virus thymidine-kinase promoter region (upstream of *Renilla* luciferase) was used as a control for transfection efficiency. The background bioluminescence level was measured in cells transfected with empty vectors (pGL3-Basic with, or without, pCDNA-3). The firefly luciferase activities were normalized to the *Renilla* luciferase activity in order to minimize experimental variability. The different fragments of the mouse neuroserpin promoter were sub-cloned to the pGL3-Basic vector containing the firefly luciferase cDNA as described previously (Berger et al., 1999). In order to fuse a DNA fragment of the neuroserpin gene with the luciferase cDNA, a 150-bp blunt-ended *Ava*I fragment (–78 to +72 relative to the transcription initiation site) was cloned to the blunt-ended *Bgl*II-*Hind*III-cut pGL3-Basic vector to produce the precursor construct pGL378bp. Then, luciferase reporter plasmids were obtained following insertion of a series of neuroserpin promoter regions in between appropriate restriction sites, leading to the generation of the following set of constructs: A (–3941 to +72), B (–2724 to +72), H (–2088 to +72), Sta (–1410 to +72), C (–1135 to +72), D (–511 to +72), E (–346 to +72), F (–125 to +72), G (–78 to +72) and –24 (–24 to +72).

Primary neuronal cultures

Primary neuronal cortical cultures were prepared from mouse embryos (E15–E16). After dissection and dissociation in Dulbecco's modified Eagle's medium (DMEM; Sigma), cortices were plated on 24-well plates coated with 0.1 mg/ml poly-D-lysine (Sigma) and 0.02 mg/ml laminin (Invitrogen). Cells were cultured in a DMEM supplement containing 5% foetal bovine serum (FBS; Gibco), 5% horse serum (Gibco) and 2 mM glutamine (Sigma). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Cytosine β-arabino-ribose furanoside (10 μmol/l) was added after 3 days in vitro (3 DIV) in order to inhibit non-neuronal proliferation. After 12 DIV, cultured neurons were treated with recombinant human BMP2, BMP4, BMP7 (R&D Systems) and AMH (Picard and Josso, 1984; Picard et al., 1986) at doses of 1 and 10 ng/ml and studied at 24 hours.

Primary astrocyte cultures

Cell cultures of cortical astrocytes were prepared from mouse pups (1–3 days after birth) using DMEM supplemented with 2 mM glutamine, 10% horse serum and 10% FBS. Every 3 days the cells (maintained at 37°C in a humidified 5% CO₂ atmosphere) were washed with a phosphate buffer saline (PBS) solution in order to eliminate microglial cells. When cultures were confluent (8–10 DIV), treatment was performed with recombinant human BMP2, BMP4, BMP7 (R&D Systems) and AMH (Picard and Josso, 1984; Picard et al., 1986) at doses of 1 and 10 ng/ml and studied at 24 hours.

Brain microvessels and primary cultures of mouse brain endothelial cells

Brain microvessel endothelial cells (BMECs) were prepared from 10-day-old or 5-week-old mice (Swiss NMRI; JANVIER, France). Cortices free from meninges and outer vessels were homogenized in a Dounce tissue homogenizer in MCDB131 supplemented with 2% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin. After centrifugation at 200 g for 10 minutes, the pellet was suspended in 18% (wt/vol) dextran solution (Sigma) and centrifuged at 2000 g for 45 minutes at 4°C. The pellet

was then suspended in MCDB131 and filtered through 70-μm-cell strainer (Falcon, BD Biosciences). The resulting filtrate was digested at 37°C in MCDB131 with 2% FBS, and 0.5 mg/ml collagenase-dispase and 100 μg/ml DNase I (Roche Diagnostics, France). The digested fragments were incubated at room temperature with anti-murine PECAM-1 (MEC 13.3; BD Pharmingen)-precoated Dynabeads (Dyna, Invitrogen) for 30 minutes in MCDB131 containing 0.1% FBS. Bead-bound microvessel fragments were collected using a magnetic particle concentrator (MPC-S; Dynal, Invitrogen) and washed three times with PBS. Thereafter, microvessels were lysed for RNA extraction or plated (to obtain MEC monolayers) onto murine collagen type IV (50 μg/ml; BD Biosciences) in MCDB131 supplemented with 10% FBS, 10% HS, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml VEGF₁₆₅ (R&D Systems), and bFGF (Invitrogen). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. After 24 hours, adherent cells were washed with PBS to remove the magnetic beads and exposed to fresh medium, which was changed every 2 days. At confluence (3–5 DIV), cells were lysed for RNA extraction. Endothelial cells derived from 10-day-old or 5-week-old mice were characterized by a cobblestone-like or spindle-shaped morphology, showed an uptake of acetylated low-density lipoprotein labelled with 1-1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) and were immunoreactive for PECAM and Von Willebrand factor.

Animals

We used neonatal (P7) and adult Swiss mice (Janvier, France); AMHR-II-deficient C57Bl6/J mice (Mishina et al., 1996), and corresponding wild-type and heterozygous littermates.

Quantitative real-time reverse-transcriptase PCR

Total RNAs were extracted from cultured cells or tissues using the NucleoSpin RNA II kit (Macherey-Nagel, France) and from the adult spinal cord with the RNeasy Lipid Tissue kit (Qiagen). RNA (1 mg) was reverse-transcribed (42°C; 1 hour) using the Promega RT system (Promega, France). The specificity of the designed primers (Beacon Designer software; Bio-Rad, France) was checked with the Blast database. The following primer sequences were used: tPA forward primer (F), 5'-CTCCGACCCATGCTCAGAA-3'; tPA reverse primer (R), 5'-TTGTA-CCAGGCCGCTGTTG-3'; neuroserpin (F), 5'-CGCCATCAATGGGATATG-3'; neuroserpin (R), 5'-CAAAGAGCGAATTGGCAAG-3'; PAI-1 (F), 5'-CGCTGCACCCTTTGAGAAAG-3'; PAI-1 (R), 5'-GGGCAGCCTGGTCATGTT-3'; protease nexin 1 (PN1) (F), 5'-TGTTGTTTCAGTGTGAAGTGCAGAA-3'; PN1 (R), 5'-CGATCAGATTTGGGGAAAGCAGAT-3'; AMH (F), 5'-ACCCTGG-TGGTGTGCCA-3'; AMH (R), 5'-AAGCGATGAGGGTCTCTAGGA-3'; AMHR-II (F), 5'-CAGGGCTGCCAGAGAAGT-3'; AMHR-II (R), 5'-TTGCTGAACACTGAGGAGACAAG-3'; cyclophilin A1 (F), 5'-CAGGGTG-GTGACTTTACACGC-3'; cyclophilin A1 (R), 5'-TGTTTGGT-CCAGCATTTGCCA-3'; β-actin (F), 5'-ATCTACGAGGGCTATGCTCTCC-3'; β-actin (R), 5'-CCACGCTCGGTCAAGATCTT-3'; GAPDH (F), 5'-CCAATGTGTCCGTCGTGGATC-3'; GAPDH (R), 5'-GCCCTCAGATGCC-TGCTTCA-3'; β2-microglobulin (F), 5'-GCCCAACATACTGAAGTCTAC-3'; β2-microglobulin (R), 5'-GCTGAAGACATATCTGACATCTC-3'. Two negative controls were performed: samples without reverse transcription, and RNase-free water instead of cDNA to control DNA contamination in samples and reagents. Assays were run using IQ SYBER Green Supermix (Bio-Rad, France) and the iCycler IQ real-time PCR detection system (Bio-Rad). Amplification conditions were: Hot Goldstar enzyme activation, 95°C for 3 minutes; 50 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The levels of expression of interest gene were computed as follows:

$$\text{relative mRNA expression} = 2^{-Ct \text{ of gene of interest}}$$

where *Ct* is the threshold-cycle value. They were also computed with respect to the mRNA expression level of the reference gene transcript using the formula:

$$\text{relative mRNA expression} = 2^{-Ct \text{ of gene of interest} - Ct \text{ of gene of reference}}$$

Immunoblotting

Cells were lysed in cold Tris-NaCl-Triton buffer containing 1% of protease inhibitor cocktail (Sigma) and were centrifuged (20 minutes; 10,000 g; 4°C). Proteins were quantified using a BCA protein assay (Pierce). Proteins (60 μg) were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with Tris-buffered saline (TBS: 10 mM Tris and 200 mM NaCl, pH 7.4) containing 0.05% Tween-20, 5% dry milk. Blots were exposed overnight to the mouse neuroserpin primary antibody G64 (1:1000; 4°C) (Lebeurrier et al., 2005), then to the goat biotin-conjugated secondary antibody (1:10,000) and finally peroxidase-conjugated streptavidin (1:5000). Proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN, France).

Single- or double-labelled immunohistofluorescence methods

Deeply anesthetized (500 mg/kg of chloral hydrate) adult mice (Swiss or C57Bl6/J) of both sexes (males *n*=10; females *n*=10) were perfused with cold heparinized saline (15 ml) and, thereafter, with a solution containing 2% paraformaldehyde, 0.2% picric

acid in 0.1 M sodium phosphate buffer pH 7.4 (150 ml). The brain and testes were removed and left in the same fixative for 18 hours. Heads from E16 embryos ($n=6$) were fixed by immersion over 18 hours. All tissues were rinsed frequently in Coons buffer containing 20% sucrose before being frozen in Tissue-Tek (Miles Scientific, Naperville, IL). Serial transversal cryostat brain sections were cut at 8 or 12 μm in a cryomicrotome. Sections were collected on gelatin-coated slides and conserved at -80°C before single- or double-label immunohistochemistry methods were applied. Briefly, a series of adjacent sections were incubated with primary antibodies to AMHR-II (1:800) (Gouédard et al., 2000); BMHR-II (1:100, Sc-5682 SantaCruz), type IV collagen (1:250, 1340-01 SouthernBiotech), NeuN (1:500, Mab377 Chemicon) and 160-kDa Neurofilament Medium (1:400, ab39371 Abcam). The specificity of the primary antibody against AMHR-II and of the secondary antibody was checked in sections from tissues in which AMHR-II expression is known to be absent (kidney, liver) and by using positive tissue samples incubated with either the secondary antibody alone, or subjected to signal extinction experiments, i.e. the combination of primary and secondary antibodies after pre-treatment with the recombinant protein used to produce the AMHR-II antiserum (recombinant form of the extracellular domain of human AMHR-II at 1 μg per ml of diluted antiserum at 1:800; data not shown). Sections were incubated at room temperature (2 hours), in a solution of donkey F(ab')₂ fragments raised against IgGs of the appropriate species and linked to fluorescein isothiocyanate (FITC, green) or tetramethyl rhodamine isothiocyanate (TRITC, red) (both 1:300, Jackson ImmunoResearch, West Grove, PA). Washed sections were coverslipped with antifade medium containing, or not, DAPI, and images were digitally captured using a Leica DM6000 microscope-coupled CoolSNAP camera and visualized with Metavue software (Molecular Devices).

Excitotoxic neuronal death

Excitotoxicity was induced by a 24-hour exposure of neuronal cultures (14 DIV) to NMDA (12.5 μM), in serum-free DMEM supplemented with 10 μM glycine. Increasing concentrations of recombinant mouse AMH (1 and 10 ng/ml) were co-applied with the excitotoxin and left in the bathing medium for 24 hours. Neuronal death was quantified by measurement of the activity of lactate dehydrogenase (LDH), using the cytotoxicity detection kit (Roche Diagnostics, Germany), released from damaged cells into the bathing medium (Koh and Choi, 1987).

Excitotoxic lesions

Excitotoxic lesions were performed under isoflurane-induced anaesthesia in male Swiss mice (25-30 g; Janvier, France). Striatal bilateral injections (coordinates: 0.5 mm posterior, \pm 2.0 mm lateral, -3.0 mm ventral to the bregma) (Franklin and Paxinos, 1997) of 10 nmol NMDA (total volume of 0.3 μl) versus NMDA + AMH (10 nmol of NMDA and 0.45 μg of AMH; total volume of 0.3 μl) were performed after placing the animals under a stereotaxic frame. Solutions were injected by the use of a micropipette made with hematologic micropipettes (calibrated at 15 mm/ μl ; assistant ref 555/5; Hecht, Sondheim-Rhoen, Germany). The needle was removed 5 minutes later.

Histological analysis of lesion volume

After 24 hours the mice were euthanized, and the brains were removed and frozen in isopentane. Cryostat-cut coronal brain sections (20 μm) were collected and stained with thionin. For volume analysis, one section out of every 10 was stained and analyzed. Regions of interest were determined through the use of a stereotaxic atlas for the mouse and an image analysis system (ImageJ) was used to measure the lesion, which corresponded to the unstained area. Results are expressed as mean \pm s.e.m.

Statistical analysis

Data are expressed as the mean \pm s.d. or s.e.m. Data were first analyzed by using the Kruskal-Wallis test, which is the nonparametric version of the ANOVA for more than two groups for unpaired series, followed by post-hoc comparisons, by using the Mann-Whitney's test, which is the nonparametric version of the *t*-test.

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