Activator of G protein signaling type 3 mRNA is widely distributed in the rat brain and is particularly abundant in the subventricular zone-olfactory bulb system of neural precursor cell proliferation, migration and differentiation

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Abstract

Activator of G protein signaling 3 (AGS3) is a guanine nucleotide dissociation inhibitor (GDI) to heterotrimeric G proteins of the G α i/o class. Previous studies have described the tissue distribution and expression changes of AGS3 in brain extracts; however the precise localization of AGS3 in intact tissue has not yet been determined. The aim of the present study is therefore to map in detail the expression of AGS3 mRNA in the rat brain by using in situ hybridization (ISH) histochemistry with a ³³P-labeled riboprobe. Hybridized sections were analyzed at the regional level after exposure to autoradiography films and at the cellular level after coating with a photographic emulsion. Our results confirm a broad distribution for AGS3 which is expressed throughout the brain. Although most regions show a low level of expression, our results reveal a high abundance of AGS3 mRNA in the cerebellum as well as in regions important for neural precursor cell proliferation (subventricular zone of the anterior lateral ventricle), migration (rostral migratory stream) and differentiation (olfactory bulb). In particular, the high abundance of AGS3 in the subventricular zone-olfactory bulb system further documents the potential role of AGS3 in neural precursor cell division, migration and/or differentiation. © 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Guanine nucleotide dissociation inhibitor; In situ hybridization; Subventricular zone; Rostral migratory stream

Activator of G protein signaling 3 (AGS3) is the first known guanine nucleotide dissociation inhibitor (GDI) for heterotrimeric G proteins [8,23]. AGS3 consists of seven tetratricopeptide repeats in the amino terminus, which is followed by a ~100 amino acid linker region connecting four ~25 amino acid GoLoco or G protein regulator (GPR) motifs. Each GPR/GoLoco motif binds to the alpha subunit of G proteins of the Gi/o class stabilizing the GDP-bound conformation of G α , thereby inhibiting G α i/o signalling [8,23,2]. The AGS3–G α i/o interaction also prevents G α i/o binding to G $\beta\gamma$ and results in the activation of G $\beta\gamma$ effector pathways [8,23,2]. This GPR/GoLoco motif was first identified in the *Drosophila* protein “loco” and it occurs either singly or as tandem repeats in a number of proteins that interact with Gi and Go class α subunits [8,17]. AGS3 has been shown

to specifically bind GDP-bound forms of all three G α i isoforms, to G α t, and weakly to G α o and G α q, but it exhibits GDI activity only toward G α i and G α t [8,2,17].

Previous Northern and Western blot studies show that full length AGS3 is primarily expressed in testes and brain, whereas a truncated form of AGS3 lacking the tetratricopeptide repeat domains is expressed in heart [8,4,19]. Within the brain, a distribution of AGS3 protein using tissue extracts from 13 different brain regions showed the presence of AGS3 throughout the brain [4]. Also, the study of primary neuronal and glial cultures indicates that AGS3 is restricted to neurons [4]. Recently, AGS3 has been shown to be upregulated in the rat prefrontal cortex and nucleus accumbens during late cocaine withdrawal and a role has been proposed for AGS3 in cocaine-induced neuroplasticity [5].

Despite these previous studies describing the tissue distribution and expression changes of AGS3 in brain extracts, the precise localization of AGS3 in intact tissue has not yet been

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determined. The aim of the present study is therefore to map in detail the expression of AGS3 mRNA in the rat brain by using in situ hybridization (ISH) histochemistry with a ^{33}P -labeled riboprobe. Our results confirm a broad distribution of AGS3 expressed at low levels throughout the brain and reveal a high abundance of AGS3 mRNA in the cerebellum as well as in the subventricular zone of the lateral ventricle (SVZa), the rostral migratory stream (RMS) and the olfactory bulb (ob).

Ten-week-old male Wistar rats weighing 190–240 g were housed in a light- and temperature-controlled room (12-h dark:12-h light cycles) with normal rat chow and water ad libitum. All efforts were undertaken to minimize animal suffering and to minimize the number of the animals used in the course of the experiments. All procedures were conducted in strict accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the animal care and use committee of Johnson and Johnson Pharmaceutical Research and Development.

Rats received a unilateral 6-OHDA or sham lesion ($N=6$ of each) of the nigrostriatal pathway by a stereotaxic injection of 8 μg 6-OHDA or solvent in the right medial forebrain bundle (coordinates 3.4 mm anterior and 1.6 mm lateral from Bregma, 8.2 mm below the cortical surface according to the atlas of Paxinos and Watson [18]), as previously described [24]. 6-OHDA is an excitotoxin which destroys midbrain dopaminergic cells which project to the forebrain. Rats were allowed to recover and were killed 28 days post-operation. The degree of lesion was determined by the loss of dopamine transporter (DAT) as visualized by DAT autoradiography in coronal sections of the forebrain at the level of the striatum (as described previously [24]).

Using primers based on a rat AGS3 (GenBank accession number AF107723) sequence, polymerase chain reaction (PCR) was performed on rat brain cDNA (BD Biosciences Clontech, Mountain View, CA, USA). Primers used were: sense primer CCT GCG GCG GCA GAG AAG CCA G, antisense primer GAA GAA ACA CTC CTC GTC AGA GGA CGG GG. The 338-bp PCR product was cloned in the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). Its sequence was confirmed on both strands in the Johnson & Johnson sequencing facility using the ABI Prism[®] BigDye[™] Terminator Cycle Sequencing Ready Reaction Kit version 2.0 (Applied Biosystems, Foster City, CA, USA) with the ABI Prism[®] 3700 DNA analyzer (Applied Biosystems). This cloned AGS3 sequence (from position 1083–1421 in the coding region of AGS3) is located in the linker region of the gene connecting a region of tetratricopeptide repeats with a region of repeated G protein regulator motifs. Linearized AGS3 plasmid was used as a template to produce sense or antisense ^{33}P -labelled riboprobes using T3 and T7 transcription systems (Promega, Madison, WI, USA), as previously described [24].

Rats were killed by decapitation. Following decapitation, brains were rapidly frozen in dry-ice cooled 2-methylbutane (-40°C) and conserved at -70°C until sectioning. 20 μm thick sections were cut out of the brains using a Leica C3050 cryostat microtome (Leica Microsystems, Wetzlar, Germany) and thaw-mounted on adhesive microscope slides (SuperFrost, Knittel Gläser, Germany). For the localization study, control

rat brains were fully sectioned in the frontal plane from rostral to caudal ($N=3$). Other control rat brains were sectioned in the sagittal planes ($N=2$). For the lesion study, unilaterally 6-OHDA lesioned brains were sectioned at the level of the caudate-putamen. The sections were then kept at -70°C .

Hybridization histochemistry was performed as previously described [24]. In brief, the sections were fixed with 4% formaldehyde, deacetylated, dehydrated and air dried at room temperature (RT). Hybridization buffer containing 6.10^6 cpm (200 kBq) of probe per 100 μl was applied to the sections. Sections were coverslipped and hybridized for 16 h at 60°C in a humid chamber. Following hybridization, sections were rinsed in SSC1x, washed in SSC2x-formamide, treated with RNase A and rinsed in SSC2x. Finally, sections were rinsed in 70% ethanol and air dried. Hybridized sections were exposed to a Kodak Biomax MR film (Kodak, Chalon, France) together with ^{14}C standard strips (ARC, St. Louis, MO, USA) for a period varying between 4 and 15 days. The slides were then immersed in Ilford K5 nuclear emulsion (Ilford, Mobberly, Cheshire, UK) diluted in water (2:1, v/v) and exposed for 4 weeks at 4°C in darkness before being developed with Kodak D-19 for 3 min at 20°C , then fixed with Kodak Readymatic. After rinsing, the sections were counterstained with methylene blue and coverslipped with aqueous medium Aquapolymount (Polysciences, Warrington, PA, USA).

Sections were exposed to films as described above. In order to correlate optical density with amount of radioactivity, ^{14}C (ARC, St. Louis, MO, USA) standards strips were co-exposed with the ISHH sections; ^{125}I (Amersham, UK) standards strips were co-exposed with the DAT autoradiography sections. Following exposure, films were developed manually in Kodak D19 and fixed with Kodak Readymatic (Kodak). Autoradiograms were subjected to densitometric analysis using an MCID M4 digital analyzer (Imaging Research, St. Catharines, Ont., Canada), as previously described [24]. The localization was identified according to the Paxinos and Watson rat brain atlas [18]. For the 6-OHDA rats, the ratio of the specific activity of the lesioned hemisphere with the specific activity of the non-lesioned hemisphere was calculated and expressed as a percentage. The one-sample *t*-test was performed using Graphpad Prism 3.02 software (Graphpad Software Incorporated, San Diego, CA, USA). Confidence with which the ratios differed from the null hypothesis was expressed as *P* values.

Illustrations for the figures were digitally recorded from autoradiograms with the MCID M4 digital analyzer and from emulsion dipped slides with the Stereo Investigator analyzing system (MicroBrightfield Inc., Williston, VT, U.S.A.) connected with a CCD camera to a Leica DMR optical microscope (magnification 40 $\times$) as bright-field photomicrographs. Illustrations were prepared with Adobe Photoshop version 5.0.2 (Adobe Systems Incorporated, San Jose, CA, USA) for contrast and brightness adjustments and cropping.

The examination of the autoradiography films hybridized with the antisense AGS3 riboprobe shows a widespread expression (Figs. 1 and 2), with all regions displaying labelling at levels higher than levels seen in corresponding regions of sections processed with sense control probe (Fig. 1). Our results show a

Fig. 1. Sagittal section overview of AGS3 mRNA distribution in the rat brain. Shown are film autoradiograms of sagittal rat brain sections upon which in situ hybridization histochemistry was performed using ^{33}P labelled AGS3 antisense (A) and sense (B) riboprobes. Sections processed with the sense probe yielded no signal as seen on film autoradiograms (B), nor on emulsion-coated tissue sections (not shown). In the lobules of cerebellum, a weak signal can be seen with the sense probe, however this signal is much lower than when the antisense probe is used. Abbreviations: Cb, cerebellum; Cx, cortex; Hip, hippocampus; Hyp, hypothalamus; IO, inferior olive; LV, lateral ventricle; ob, olfactory bulb; RMS, rostral migratory stream; Str, striatum. Scale bar: 5 mm.

similar AGS3 mRNA expression pattern in all five brains analyzed (three brains sectioned frontally and two brains sectioned sagittally).

Moving rostral to caudal, dense AGS3 expression is seen in all cellular layers of the olfactory bulb (Fig. 2A). Dense expression is also observed in the rostral migratory stream (Figs. 1A, 2A and 2B), while low expression is observed in the neocortex and moderate expression in the olfactory tubercle and piriform cortex (Figs. 1A, 2A and 2C). At the level of the striatum, low to moderate AGS3 levels are found in the caudate-putamen, nucleus accumbens and globus pallidus while dense expression is seen in the subventricular zone (Fig. 2C). In the thalamus, dorsolateral portions show low AGS3 levels. Medial and ventrolateral nuclei of the thalamus such as the parafascicular nucleus, the zona incerta and the ventrolateral geniculate nucleus show moderate AGS3 levels (Fig. 2E). AGS3 shows moderate expression in amygdalar nuclei (Fig. 3E) and low to moderate expression in the different fields of the hippocampus (Fig. 2E and F). In the midbrain, moderate AGS3 levels are found in the ventral tegmental area, substantia nigra pars compacta and dense levels are seen in the red nucleus (Fig. 2F). In caudal sections of the brain, dense AGS3 expression can be seen in the cerebellum and peritrigeminal zone (Fig. 2G) as well as in the inferior olive (Fig. 1A).

At the cellular level, examination of emulsion-coated tissue sections revealed specific silver grain clusters overlying methylene blue counterstained cells (Fig. 3). In more detail, AGS3

Fig. 2. Detailed illustration of AGS3 mRNA distribution in the rat brain. Autoradiograms of rat brain sections hybridized with ^{33}P labelled AGS3 antisense riboprobes in a sagittal plane at the level of the olfactory bulb (A), and in the frontal planes at the level of the prefrontal cortex (B), striatum (C), globus pallidus (D), thalamus/hippocampus (E), mesencephalon (F) and pons/cerebellum (G). Abbreviations: ***, third ventricle; 3, oculomotor nucleus; Acb, accumbens nucleus; BL, basolateral amygdaloid nucleus; BM, basomedial amygdaloid nucleus; CA1, field CA1 of hippocampus; CA3, field CA3 of hippocampus; CPu, caudate putamen; DG, dentate gyrus; EPL, external plexiform layer; fmi, forceps minor of the corpus callosum; GCL, granule cell layer; GL, glomerular layer; GP, globus pallidus; La, lateral amygdaloid nucleus; LC, locus coeruleus; MCL, mitral cell layer; P5, peritrigeminal zone; PF, parafascicular thalamic nucleus; Pir, piriform cortex; PMCo, posteromedial cortical amygdaloid nucleus; PVN, paraventricular hypothalamic nucleus; R, red nucleus; RMS, rostral migratory stream; SEZ, subependymal zone; SNc, substantia nigra pars compacta; SVZa, subventricular zone; Tu, olfactory tubercle; VLG, ventral lateral geniculate nucleus; VTA, ventral tegmental area; ZI, zona incerta. Scale bar: 1.5 mm (A) and 5 mm (B–G).

Fig. 3. Photomicrographs of emulsion-dipped sections depicting the cellular mRNA expression of AGS3 in the rat brain (bright field microscopy, counterstained with methylene blue). Silver grains elicited by the ^{33}P labeled AGS3 antisense probe hybridized to the section appear as black grains. Photomicrographs were taken at the level of: the granular layer of the olfactory bulb (ob), the prefrontal cortex (PFC), the subventricular zone (SVZa), the ventral lateral geniculate (VLG), the third ventricle (D3V) and the cerebellum (Cer, granular layer is left of dashed line). (**) Lateral ventricle; (***) third ventricle. Scale bar: 20 μm .

positive cells are seen in all cellular layers of the olfactory bulb (shown for the granular cell layer, Fig. 3, ob). Subventricular expression was restricted to the lateral ventricle (Fig. 3, SVZa), with no AGS3 mRNA found in cells lining other ventricles, such as illustrated for the third ventricle (Fig. 3, D3V). In the subventricular zone of the lateral ventricle, a fraction of the cells showed no AGS3 mRNA (Fig. 3, SVZa, some methylene-blue positive cells not showing silver grain clusters). In the cerebellum, AGS3 positive cells were found in the granular cell layer (Fig. 3, Cer).

To test whether dopamine denervation influences its expression, AGS3 expression levels were measured at the level of the striatum in unilaterally 6-OHDA lesioned rats. Only those rats having a degree of lesion of greater than 95% (as determined by reduction in striatal dopamine transporter levels) were included in the analysis. When AGS3 mRNA levels of 6-OHDA lesioned rats were compared to sham lesioned rats, no significant modifications of AGS3 expression levels were found in the caudate-putamen, nucleus accumbens nor in the subventricular zone ($+9.0 \pm 4.6\%$, $+10.8 \pm 3.1\%$, $-2.1 \pm 4.5\%$, respectively; mean $\pm$ S.E.M., $P > 0.05$ for all regions).

In the present report, we show for the first time the distribution of AGS3 mRNA in intact rat brain tissue. The specificity of the in situ signals presented here is ensured by probe design and verified by processing sections with sense AGS3 probe. The probe was generated using the only significant stretch of the AGS3 sequence which does not contain repeats, i.e. the linker region between the seven tetratricopeptide repeats and the four GPR/GoLoco motifs [23]. For all brain regions, background levels as determined by the incubation of sections with sense probe are consistently lower than levels seen with antisense probe, indicating a widespread expression of AGS3 in the rat brain.

These results are consistent with a previous report showing that the distribution of AGS3 protein as determined by western blot of brain homogenates is uniform throughout the brain [4].

The neuroanatomical distribution of AGS3 described here fits with the distribution of its primary interaction partners, the G protein alpha subunits $\text{G}\alpha\text{i}1$, $\text{G}\alpha\text{i}2$, $\text{G}\alpha\text{i}3$, $\text{G}\alpha\text{o}$ and $\text{G}\alpha\text{q}$. Indeed, neuroanatomical distributions of these five primary interaction partners of AGS3 have previously been described and, taken together, they cover all brain regions [6,7,11,15,25], as does AGS3. Nevertheless, the specific distribution of each of these five $\text{G}\alpha$ subunits indicates that certain AGS3- $\text{G}\alpha$ interactions would predominate in certain brain regions.

AGS3 mRNA is highly abundant in the granule cells of the cerebellum, where several AGS3 interaction partners can also be found. Indeed, these cells express $\text{G}\alpha\text{i}1$, $\text{G}\alpha\text{i}2$, $\text{G}\alpha\text{o}$ and $\text{G}\alpha\text{q}$ [6,11,15,25]. Therefore, cerebellar AGS3 may be involved in regulating signaling cascades mediated by any one of these four $\text{G}\alpha$ subunits. Because of these many theoretical possibilities for interaction, the role of AGS3 in the cerebellum appears diffuse. However, images from emulsion dipped sections (Fig. 3) reveal an uneven cellular distribution of AGS3 in the cerebellum, with certain granular cells showing abundant AGS3 levels and others with no or low AGS3 expression. Further studies will be necessary to determine whether AGS3 co-localizes preferentially with any of the abovementioned $\text{G}\alpha$ subunits.

An important finding of our study is the demonstration of the enriched AGS3 expression in the subventricular zone of the anterior lateral ventricle (SVZa), the rostral migratory stream and the olfactory bulb. Together with the subgranular layer of the dentate gyrus, the SVZa is one of the few regions of the brain which maintains a significant proliferative capacity into adult-

hood [12]. Neural progenitors generated in the SVZa migrate in a concise pathway known as the rostral migratory stream (RMS) to the olfactory bulb (ob) where they differentiate into neurons [16]. Much attention is currently focussed on the functioning of the SVZa-RMS-ob system as it is an important reservoir of progenitor cells in the adult brain which could be used for neuroregenerative therapies if the molecular switches for activating it were known. The strikingly high abundance of AGS3 in the SVZa-RMS-ob system shown here indicate that AGS3 may be just one such molecular switch. Interestingly, one of the common interaction partners of AGS3, G α i2, is also enriched in the SVZa and RMS [1], indicating that the AGS3-G α i2 interaction may be predominant in this region. Furthermore, Gi2 signaling has previously been shown to be involved in proliferation of neural progenitors [22] and it is therefore feasible that AGS3 may regulate SVZa-RMS activity via its regulatory action on G α i2. Complementary research is necessary to verify this hypothesis and clarify the exact mechanism of action.

Previous studies of AGS3 function in brain or in unicellular systems fit with its potential role as a regulator of cell division, cell survival or neural plasticity in the SVZa-RMS-ob system. For instance, AGS3 orthologs from *D. melanogaster* and *C. elegans* have been shown to be required for events in asymmetric cell division of neuroblasts [13,21]. AGS3 is also closely related to another protein called LGN (Leu-Gly-Asn enriched protein), which is also implicated in regulating key aspects of cell division and cell polarity [10], and mammalian LGN can functionally substitute for the loss of the AGS3/LGN ortholog in *Drosophila* [26]. Also, in a yeast two hybrid screen of AGS3 interactors, Blumer and co-workers identified the serine threonine kinase LKB1 which is involved in cell polarity and cell cycle progression and which is capable of phosphorylating AGS3 [3].

Although there are but few reports of AGS3 function in the mammalian brain, some in vivo results also hint at a role for AGS3 in neural precursor proliferation, migration and/or differentiation. Most recently, AGS3 has been shown to control spindle orientation and asymmetric cell fate of cerebral cortical progenitors of the developing mouse brain [20], although these properties remain to be studied in adult mammalian brains. In other studies, AGS3 has been shown to be upregulated in the prefrontal cortex and nucleus accumbens during late withdrawal from chronic cocaine self administration in rats [5]. Interestingly, this upregulation is observed only 3–8 weeks after withdrawal, but is not observed at 1 week following withdrawal. These time points of 3–8 weeks are in the range of those needed to stimulate proliferation and migration of neural precursors [9]. It would be interesting to investigate whether the upregulation of AGS3 in cocaine withdrawal would be due to the activation of neural precursors from the subventricular zone which then migrate to the PFC or nucleus accumbens or if AGS3 is upregulated in pre-existing neurons. Because of the potential link between AGS3 and SVZa-RMS-ob activity and given recent reports showing that deletion of dopaminergic nigrostriatal projections to the SVZa impairs precursor cell proliferation in the SVZa [14], we decided to test whether AGS3 expression would be modulated by dopamine depletion. However, this was not the case in our experimental groups of rats showing depleted

dopamine levels following 6-OHDA lesion (data not shown). Future studies should further verify the potential link between dopamine levels and a role for AGS3 in SVZa activity at the level of protein expression or posttranslational modifications or at other time points following lesion.

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