Rapid Communication

Fatty Acid Amide Hydrolase, the Degradative Enzyme for Anandamide and Oleamide, Has Selective Distribution in Neurons Within the Rat Central Nervous System

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Fatty acid amide hydrolase (FAAH) is a membrane-bound enzyme activity that degrades neuromodulatory fatty acid amides, including oleamide and anandamide. A single 2.5-kb FAAH mRNA is distributed throughout the rat CNS and accumulates progressively between embryonic day 14 and postnatal day 10, remains high until postnatal day 30, then decreases into adulthood. FAAH enzymatic activity, as measured in dissected brain regions, was well correlated with the distribution of its messenger RNA. In situ hybridization revealed profound distribution of FAAH mRNA in neuronal cells throughout the CNS. The most prominent signals were detected in the neocortex, hippocampal formation, amygdala, and cerebellum. The FAAH distribution in the CNS suggests that degradation of neuromodulatory fatty acid amides at their sites of action influences their effects on sleep, euphoria, and analgesia. J. Neurosci. Res. 50:1047–1052, 1997.

INTRODUCTION

The enzyme fatty acid amide hydrolase (FAAH) has been shown to degrade fatty acid amides (Cravatt et al., 1996) such as oleamide, a lipid isolated from the cerebrospinal fluid of sleep-deprived cats (Lerner et al., 1994; Cravatt et al., 1995), and anandamide, the putative endogenous ligand for the cannabinoid receptor (Devane et al., 1992). In addition to activation of the cannabinoid receptor, anandamide has also been shown to modulate 5HT3 receptor-mediated responses in rat nodose ganglion neurons (Fan, 1995). Since its identification and isolation, oleamide has been shown to have diverse sleep-inducing (Cravatt et al., 1995) and neuromodulatory properties, including potentiation of 5HT3 receptor-mediated responses in frog oocytes (Huidobro-Toro and Harris, 1996) and antagonism of gap junction communication in cultured glial cells (Guan et al., unpublished observations). Hence, oleamide appears to be a new member of a family of amidated lipid compounds that can act as neuromodulators.

FAAH rapidly hydrolyzes oleamide into its inactive acid, oleic acid, in rat membranes and converts anandamide into arachidonic acid at a similar rate (Cravatt et al., 1996). The human and mouse homologs of FAAH have also been recently cloned (Giang and Cravatt, 1997). Hence, FAAH is likely to be responsible for rapid signal termination of these endogenous signaling molecules in mammals, including humans. The identification of an enzyme activity specific for fatty acid amides represents an important step toward understanding the metabolism, regulation, and functional significance of this growing family of bioactive lipid modulators. In the present study, we have determined the localization and cellular distribution of FAAH mRNA using Northern blot and in situ hybridization analysis and have correlated FAAH expression with its amidase activity in rat CNS.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200–250 g) were used for all experiments. Rats were maintained on a 12 hr

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light/dark schedule before sacrifice. Food and water were available ad libitum.

**Northern (RNA) Blot Analysis**

Northern blots were prepared using 2 μg poly(A+) RNA from selected adult rat brain regions and a few peripheral tissues. Developmental Northern blots were prepared using 2 μg poly(A+) RNA from whole brain of rats aged embryonic day 16 to adult. For embryonic day 14, mRNA from whole head was used. The RNA transcripts were fractionated by electrophoresis on a 1.5% agarose gel containing formaldehyde, transferred to a biotrans membrane by the method of Thomas (1980), and prehybridized for 30 min in Expresshyb (Clonetech, Palo Alto, CA). An 800 bp insert (25–100 ng) of FAAH was labeled with [α-32P]-d CTP by oligonucleotide labeling (Feinberg and Vogelstein, 1983) to specific activities of approximately 5 × 10⁸ cpm/μg, added to the prehybridization solution, and incubated 1 hr. Filters were washed to high stringency (0.2× SSC) (1× SSC: 0.015 M NaCl and 0.0015 M Na citrate) at 68°C then exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY) for up to 1 week.

**FAAH Enzymatic Assay**

Male Sprague-Dawley rats (n = 4) were sacrificed by decapitation, and brains were removed immediately, dissected into subregions, and placed in ice-cold PBS. Brain regions were homogenized in 1 mM NaHCO₃ and assayed for fatty acid amide hydrolase activity as follows: 14C-oleamide (100 μM; in ethanol) (50 μCi/μmol; see Cravatt et al., 1995 for synthesis) was incubated at 37°C for 1 hr with 40 μl of protein (50 μg) and 156 μl of 125 mM Tris-HCl, pH 9.0 (final ethanol concentration of 2.0%). The reaction mixture was then partitioned between ethyl acetate (1.0 ml) and 0.07 M HCl (0.6 ml). The ethyl acetate layer was concentrated under a constant stream of gaseous N₂, and the remaining residue was resuspended in 15 μl of ethanol. Products were separated by thin-layer chromatography (60% ethyl acetate in hexanes), eluted from silica with scintillation fluid, and quantified by scintillation counting. Each brain region was assayed from two different animals in triplicate.

**In Situ Hybridization Analysis**

In situ hybridization was performed on free-floating sections as described previously (de Lecea et al., 1996). Coronal sections, 25 μm thick, or sagittal sections, 40 μm thick, from adult rats (n = 4) were hybridized at 55°C for 16 hr with 35S-labeled single-stranded antisense RNA probes at 10⁷ cpm/ml. 35S-Labeled probes were synthesized from a 1-kb fragment from the 3’ end of the cDNA clone using the Maxiscript Transcription Kit (Ambion, Austin, TX). Excess probe was removed with 2× SSC containing 14 mM β-mercaptoethanol (30 min), followed by incubation with 4 μg/ml ribonuclease A in 0.5 M NaCl, 0.05 M EDTA, and 0.05 M Tris-HCl (pH 7.5) for 1 hr at 37°C. Stringency washes were carried out at 55°C for 2 hr in 0.5× SSC, 50% formamide, and 0.01 M β-mercaptoethanol, and then at 68°C for 1 hr in 0.1× SSC, 0.01 M β-mercaptoethanol, and 0.5% sarcosyl. Slices were mounted onto gelatin-coated slides and dehydrated with ethanol and chloroform before autoradiography. Slides were exposed 1–4 days to Kodak X-AR film and then dipped in Ilford K-5 emulsion; after 4 weeks, they were developed with Kodak D19 developer, fixed, and counterstained with Richardson’s blue.

**RESULTS**

**Regional and Developmental Expression of FAAH mRNA in Rat Brain**

To examine the regional distribution of FAAH mRNA expression, Northern blot analysis was performed using poly(A)+ mRNA from eight dissected regions of the adult rat brain and selected peripheral tissues. A single 2.5-kb band, which corresponds to that reported previously (Cravatt et al., 1996), was detected to various extents in all brain regions examined (Fig. 1A). A relatively high level of expression was detected in hippocampus, which approached the level observed in the liver, the tissue in which FAAH was originally isolated. Moderate levels of expression were detected in cerebellum, thalamus, and hypothalamus, with the lowest levels detected in the brainstem and pituitary. The expression pattern of FAAH mRNA during development was also determined using poly(A)+ RNA from whole brain of rats aged embryonic day 14 to adult. FAAH expression appeared weakly at embryonic day 14 and increased progressively until postnatal day 10, at which maximal hybridization was observed (Fig. 1B). FAAH expression remained at this high level until postnatal day 30, then decreased with age until adult (60 days) (Fig. 1B).

**Regional Localization of FAAH Hydrolase Activity in Rat Brain**

The ability of FAAH to hydrolyze oleamide into oleic acid was measured in homogenates of dissected regions of the adult rat brain, including cerebral cortex, hippocampus, hypothalamus, striatum, thalamus, cerebellar cortex, brainstem, pituitary, and whole brain. Membrane fractions were incubated with 14C-labeled oleamide (100 μM) for 1 hr at 37°C, and conversion to 14C-labeled oleic acid was determined. The highest level of enzyme activity was measured in homogenates of the hippocampus and cortex (approximately 2 pmol product/min-ng),
whereas the lowest levels, (approximately 0.4 pmol product/min-mg), were detected in brainstem and pituitary (Fig. 2). Intermediate activities were measured in cerebellum, olfactory bulb, thalamus, and striatum. In Situ Hybridization of FAAH Transcripts in Adult Rat Brain

To determine anatomical distribution of FAAH further, in situ hybridization was performed using an antisense 35S-labeled riboprobe from an FAAH cDNA clone. Specific hybridization signals of FAAH mRNA were widespread throughout the CNS of the adult rat. All sections were subsequently dipped in autoradiographic emulsion, in order to detect cellular localization of FAAH mRNA.

The most abundant signals were detected in neurons of the neocortex, hippocampus, amygdala, and cerebellar cortex. FAAH expression was predominant in the neuronal cell bodies of all cortical layers, except layer I (Figs. 3, 4). FAAH mRNA was uniformly expressed in all regions of the neocortex, including motor, somatosensory, and visual areas. Film autoradiographs showed abundant FAAH hybridization in the hippocampal formation. After emulsion dipping, it was apparent that FAAH mRNA was localized to pyramidal and nonpyramidal cells of the CA1, CA2, and CA3 subfields and the granule cells of the dentate gyrus (Fig. 4). Prominent hybridization signals were detected in the amygdala, primarily the lateral, basolateral, and basomedial amygdaloïd nuclei. In the cerebellum, abundant hybridization signals for FAAH mRNA were observed in the Purkinje and granule cell layers (Figs. 3, 4). Additionally, strong signals were detected in piriform cortex, the anterior olfactory nuclei, olfactory tubercle, and islands of Calleja (Fig. 3). FAAH

Fig. 1. Northern blot analysis of FAAH mRNA distribution in rat brain Northern blots with 2 µg poly (A)1-selected RNA from (A) whole brain, olfactory bulb, cerebral cortex, hippocampus, striatum, hypothalamus, thalamus, cerebellum, pituitary, liver, kidney, and heart and (B) whole brain of rats at the indicated number of days of embryonic (E) or postnatal (PN) development, and adult (60 days), using an internal 800-bp fragment from an FAAH cDNA clone as a probe. For embryonic day 14, mRNA from whole head was used. A cyclophilin probe was included in the series as a control for comparable blot loading and RNA integrity. Only the regions of the blots containing the hybridized signals are shown.

Fig. 2. Distribution of fatty acid amide hydrolase activity in adult rat brain assays were carried out in freshly prepared homogenates of whole brain, cerebral cortex, hippocampus, hypothalamus, striatum, thalamus, cerebellum, brainstem, and pituitary (1.25 mg/ml protein). Hydrolase activity was determined by measurement of conversion of 14C-labeled oleamide (100 µM) into 14C-oleic acid during a 60-min incubation at 37°C. Each assay was performed two times, in triplicate. Error bars represent standard deviation of average values.
expression was also localized to the caudate-putamen, where it appeared to have a homogeneous distribution, suggesting localization to medium spiny neurons, whereas globus pallidus showed weaker hybridization. In the hypothalamus, FAAH expression was detected in the ventromedial hypothalamic nucleus and the subthalamic nucleus (Fig. 3), and in the thalamus, FAAH mRNA seemed to have a homogeneous partitioning throughout most of the thalamic nuclei. Messenger RNA for FAAH was confined to very few regions in the brainstem. The most intense FAAH mRNA hybridization was detected in the pontine nuclei (Fig. 3), with lower hybridization signals observed in the medial superior and inferior olives. A weak signal was observed over corpus callosum and anterior commissure, where glial cells are predominant, indicating that FAAH expression may occur in non-neuronal cells, in addition to neurons. The sense-strand RNA probe of FAAH failed to give a detectable signal in these brain regions (data not shown). All FAAH hybridization signals were abolished in RNase (100 µg/ml) pretreated sections (data not shown).

DISCUSSION

In the present study, we have determined the distribution of fatty acid amide hydrolase (FAAH) and its mRNA in rat CNS. There was generally good correlation observed between the distribution of FAAH mRNA in the brain, as indicated by Northern blot analysis, and the hydrolytic properties of the enzyme in the nine selected brain regions. The localization of FAAH activity in the CNS reported in the present study also agrees with previous reports on regional anandamide hydrolase activity in rat brain (Hillard et al., 1995). During development, our data indicate that FAAH expression increases dramatically during the postnatal developmental stages of the rat
brain, a time at which synapse formation is occurring at a rapid pace (Eayrs, 1966). This temporal expression pattern may suggest that these lipid substrates are not required until the CNS has achieved an advanced state of development or that these molecules could have detrimental effects if present at earlier embryonic stages.

At higher resolution, FAAH was shown to be widely expressed in neurons throughout the brain. The most prominent hybridization signals were detected in the neocortex and several limbic regions of the brain, primarily the amygdala, hippocampus, and olfactory tubercle. FAAH mRNA was also found in the pontine nuclei and nuclei of the hypothalamus. These regions have been implicated in sleep activity and motivational and emotional states; thus, our data suggest that FAAH may regulate the levels of lipid molecules that influence these processes. This observation agrees with the known behavioral effects (euphoria, hallucinations, analgesia) associated with cannabinoid receptor activation in humans and the sleep-inducing properties related to oleamide in rats. FAAH transcripts are also likely found in some glial cells, as indicated by the low levels of detection in corpus callosum and anterior commissure.

FAAH has been shown to degrade oleamide into its inactive acid, oleic acid, and anandamide into ethanolamine and arachidonic acid, which does not have cannabinoid-like activity (Cravatt et al., 1996). Hence, FAAH is likely to be responsible for the endogenous signal inactivation of these neuromodulatory fatty acid amides. Oleamide has been shown to potentiate 5HT₂ receptor-mediated responses in cultured cells (Huidobro-Toro and Harris, 1996) and modulate 5HT₇ receptor-mediated effects in transfected HeLa cells (unpublished studies); in addition to activation of cannabinoid receptors, anandamide has also been shown to inhibit 5HT₃ receptor-induced responses (Fan, 1995). Accordingly, the high-

Fig. 4. In situ hybridization of FAAH mRNA is detected neuronal populations of adult rat brain. Photomicrographs in darkfield (A–C,E) and brightfield (D,F) illumination of selected brain regions are shown. Strong hybridization signals are detected in dentate gyrus (DG) of the hippocampus (A), amygdala (B), layers II–VI of cerebral cortex (C,D) (layers shown from right to left in panel C), and granule (Gr) and Purkinje (P) layers of the cerebellum (E,F). Mol, molecular cell layer. Arrows indicate positively labeled pyramidal cells in neocortex (D) and Purkinje cells in cerebellum (F). Emulsion-dipped sections were counterstained with Richardson’s blue. A–C,E, ×10; D,F, ×40.
resolution distribution of the mRNA of these 5HT receptor subtypes in target tissues shows several overlapping expression patterns with that of FAAH mRNA in rat CNS. 5HT2 and 5HT3 receptor mRNA is localized in neocortex, piriform cortex, olfactory bulb, and amygdala (Tecott et al., 1993; Wright et al., 1995), all of which are enriched in FAAH mRNA. Additionally, 5HT3 mRNA is found in the hippocampal formation, and 5HT2 mRNA is located in the pontine nuclei (Tecott et al., 1993; Wright et al., 1995). 5HT7 Receptor transcripts are localized in more discrete brain regions, and overlapping signals with FAAH expression include the hippocampus and various thalamic nuclei (Lovenberg et al., 1993). In addition, the localization of cannabinoid receptor is similar to that of FAAH mRNA hybridization, including abundant expression in neocortex, the hippocampus, amygdala, hippocampus, and cerebellum (Mailleux and Vanderhaeghen, 1992). Like FAAH, the cannabinoid receptor mRNA is also detected at low levels throughout the brainstem, but is abundant in the pontine nuclei (Mailleux and Vanderhaeghen, 1992).

FAAH mRNA and its corresponding hydrolase activity are localized in numerous, yet discrete regions of rat brain, as are the cannabinoid and some 5HT receptor mRNAs. As for other neurotransmitter metabolizing enzymes (e.g., acetylcholinesterase), such parallel distribution supports the likelihood that FAAH participates in endogenous inactivation of such neuromodulatory fatty acid amides at their site of action and, thus, influences the activities of molecules such as oleamide and anandamide on sleep, euphoria, and analgesia.

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REFERENCES


