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Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor subunit expression in rat olfactory bulb

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Abstract

The alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (AMPARs) mediate rapid responses at most central excitatory synapses, including those in the olfactory bulb (OB). These receptors are composed of the glutamate subunits GluR1–4, which each has two splice variant (flip/flop) forms. We recently showed that AMPARs on OB neurons are kinetically and pharmacologically diverse. Here, we explored whether this functional heterogeneity reflects a diverse expression of AMPAR subunits and/or splice variants. Total RNA from rat OBs was amplified by RT-PCR. Digestion of the panGluR PCR product with subunit-specific restriction enzymes revealed that the OB expresses mRNAs for GluR1–4 but in different relative amounts i.e., GluR2 ($61 \pm 2.4\%$), GluR1 ($31 \pm 3.5\%$), GluR4 ($6.3 \pm 1.4\%$), GluR3 ($1.4 \pm 0.7\%$). Furthermore, GluR2 and GluR4 transcripts were composed of similar amounts of flip and flop, whereas GluR1 and GluR3 transcripts consisted mostly of flip. If similar to other brain regions, this heterogeneity in patterns of expression may facilitate information processing.

Keywords: GluR subunits; Flip; Flop; Glutamate; RT-PCR; Olfaction; AMPA receptor

The alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of ionotropic glutamate receptor mediates rapid responses at the majority of excitatory synapses in the CNS [7], including those in the olfactory bulb (OB) [3,34]. These receptors are heterotetramers composed of various combinations of four subunits: GluR1–4 [27]. This heterogeneity of subunit composition [5,14,22], along with various forms of post-transcriptional modification [8,9,15,17,30–32], markedly influence AMPA receptor (AMPAR) function. We recently reported that AMPARs expressed on OB neurons are kinetically and pharmacologically diverse, with varying rates and extent of receptor desensitization and sensitivity to the AMPAR modulator cyclothiazide [4], a drug that reduces AMPAR desensitization [24,25]. This functional diversity led us to hypothesize that the OB expresses a heterogeneous array of AMPAR subunits and splice variants.

The functional properties of AMPARs in a given neuron are largely determined by which genes are expressed. Most AMPAR subunits undergo two types of post-transcriptional modification, RNA editing and alternative splicing, which contributes to a high degree of structural and functional diversity. For example, GluR2 undergoes RNA editing at the "Q/R" site. The single-codon, glutamine (Q)-to-arginine (R) substitution in GluR2 dramatically alters the channel's current–voltage relationship [33] and reduces calcium permeability [6,12,35].

Alternative splicing of the AMPAR gene generates two splice variants of each GluR subunit, referred to as "flip" and "flop" [9,15,31]. The variant region, the flip/flop domain, is due to differences in a 38-amino acid sequence that forms part of the TM3–TM4 loop [9,15,31]. Although the physiological significance of flip and flop is unclear, AMPARs composed of different splice variants show obvious differences in both their rates and extents of desensitization [8,22] and sensitivity to cyclothiazide [24,25].

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Recent immunohistochemical findings indicate that all AMPAR subunits are expressed in the OB [21], but the quantities of individual subunits remain unclear. In the present study, we used RT-PCR to quantify the relative abundance of GluR1–4 in the OB, including their splice variant (flip/flop) forms. Our findings indicate that all GluR1–4 subunits and their flip/flop isoforms are expressed in the OB, but in varying amounts. Heterogeneity in patterns of AMPAR subunit and splice variant expression may underlie the kinetic and pharmacologic diversity of AMPARs in the OB [4] and facilitate olfactory information processing.

All animals were used in accordance with the institutional guidelines of 'The Care and Use of Laboratory Animals' approved by the National Institutes of Health and The Florida State University's Animal Care and Use Committee.

Total mRNA was purified from individual 5-day-old rat olfactory bulbs ($n = 5$ rats; 10 bulbs) using the Trizol extraction method (GibcoBRL); 30 μg of total RNA was extracted from each bulb as assayed by spectrophotometry. Single-stranded cDNA was synthesized from 15 μg of the total RNA in a 20 μl RT reaction with Superscript II (GibcoBRL) and an oligodT-T7 primer.

Amplification of the AMPAR subunits was performed on 1 μl of olfactory bulb RT reaction product in a 50 μl volume using PCR Platinum Super Mix (GibcoBRL) and panGluR primers (200 nM) designed to recognize all four AMPAR subunits [1,16] (see Table 1). Cycling conditions were 92 °C for 20 s, 56 °C for 30 s, and 72 °C for 45 s for 25 cycles, with a final 5 min extension at 72 °C in a MJ Research PTC-200 thermal cycler. To quantify the relative proportions of specific subunits within the amplified cDNA, equal volume aliquots (10 μM) of the panGluR PCR product were digested in parallel at 37 °C for 3 h with restriction enzymes that cleaved specific subunits: Bgl I for GluR1; Bsp 1286 I for GluR2, Sal I for GluR3; and EcoR I for GluR4 (see Table 2) [1,16]. Following the digestion, the enzyme-cleaved products were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide.

Aliquots of the panGluR PCR were purified (Qiagen) and reamplified using HotStarTaq (Qiagen) with primer pairs (200 nM) specific for each AMPAR subunit and bracketing the flip/flop variant region. Cycling conditions were 35 cycles of 92 °C for 20 s, 30 s of annealing and 72 °C for 45 s. Annealing temperatures were optimized for each primer pair. The subunit-specific PCR products were digested with enzymes specific for either flip or flop variants: Msp I for GluR1 flip, Ava I for GluR2–4 flip, Hph I for GluR1 flop, and Hpa I for GluR2–4 flop (see Table 3) [1,16]. Digested fragments were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

Table 2
Enzymes and digestion products that distinguish AMPA receptor subunits

Subunit	Enzyme	Digest sizes (bp)
GluR1	Bgl I	300/449
GluR2	Bsp 1286 I	478/271
GluR3	Sal I	359/396
GluR4	EcoR I	411/338

Table 3
Enzymes and digestion products that distinguish flip/flop isoforms

Subunit	Flip		Flop	
	Enzyme	Digest sizes (bp)	Enzyme	Digest sizes (bp)
GluR1	Msp I	722/69	Hph I	615/176
GluR2	Ava I	519/207	Hpa I	426/300
GluR3	Ava I	409/330	Hpa I	496/243
GluR4	Ava I	518/202	Hpa I	425/295

The proportion of subunits and flip/flop variants were quantified using the Gel Doc digital imaging system and QuantityOne analysis software (BioRad). For each individual restriction digest, the size (area × intensity − local background) of the lower-molecular weight bands representing digested DNA was expressed as a proportion of total DNA (digested + undigested bands). Because of variability in the efficiency of digestion of the panGluR product into the four individual subunits, the overall proportion of subunits for each individual rat was normalized to 100; normalized proportions for each subunit were then averaged across rats. For flip and flop variants, the proportion of flip and flop in each subunit of each rat was averaged across individual rats.

Total RNA isolated from P5 rat olfactory bulbs ($n = 5$ rats; 10 bulbs), as well as a homologous region of the cDNA from the GluR1–4 subunits, was amplified by RT-PCR. The relative abundance of each subunit was determined by digesting the panGluR PCR product using subunit-specific restriction enzymes. Our results indicate that all of the GluR1–4 subunit mRNAs are expressed in the OB (see Fig. 1A). Restriction analysis indicated that the relative abundance of the subunit mRNAs was GluR2 ($61 \pm 2.4\%$) > GluR1 ($31 \pm 3.5\%$) > GluR4 ($6.3 \pm 1.4\%$) > GluR3 ($1.4 \pm 0.7\%$) (see Fig. 1B). Each of these proportions was statistically different from the other, with GluR2 greater than GluR1 ($p = .0004$); GluR1 greater than GluR3 ($p = .00006$); GluR1 greater than GluR4

Table 1
Primers and annealing temperatures for PCR amplification of AMPA receptor subunits

Subunit	Product size (bp)	Sense primer	Anti-sense primer	Annealing (°C)
panGluR	748 (1, 2, 4); 754 (3)	CCTYTRgCYTATgARATCTggATgTg	TCgTACCACCATTTgTTTTTCA	56.0
GluR1	791	CAACAgCCTgTggTTCTCCC	CATTgATggATTgCTgTggg	69.0
GluR2	726	TggTggTTCTTTACCCTg	TgCAAAATTCTgggAATTC	61.6
GluR3	739	gggTgCTTTCATgCAgCAAg	gAgTTTCATgCgTTTggACTC	69.7
GluR4	720	ggTggTTCTTCACACTCATC	CACTCCCAgTgATggATAAC	65.0

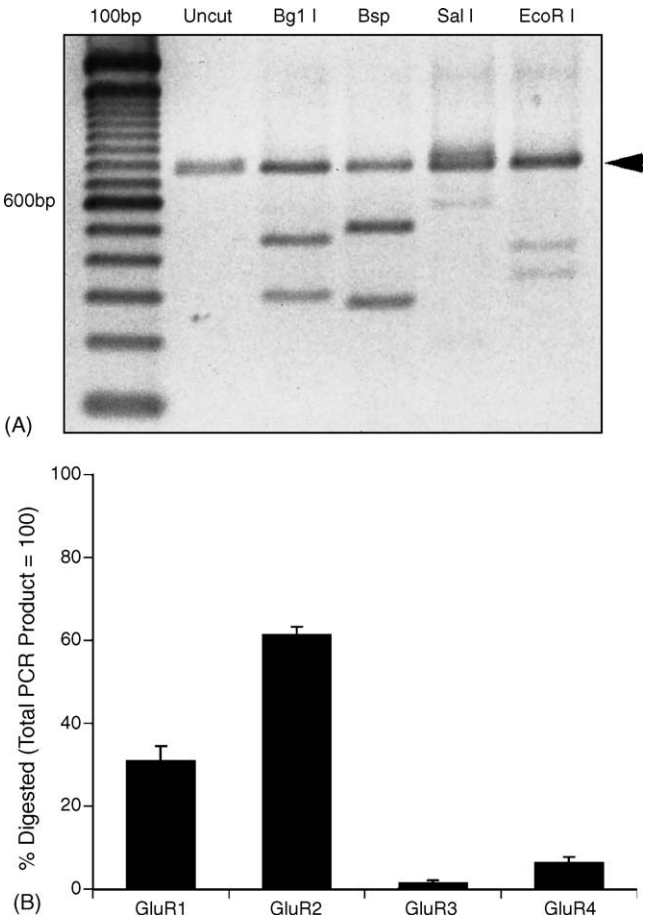

Fig. 1. (A) Example of subunit-specific restriction digest of panGluR RT-PCR product amplified from rat olfactory bulb RNA. Arrow on the right indicates position of panGluR product. Uncut: control undigested PCR product; Bgl I: GluR1-specific digest; Bsp: GluR2-specific digest; Sal I: GluR3-specific digest; EcoR I: GluR4-specific digest. (B) Relative proportion of AMPA receptor subunits from quantification of digested and undigested electrophoretic bands. Bars indicate mean ± S.E.M. for five rats.

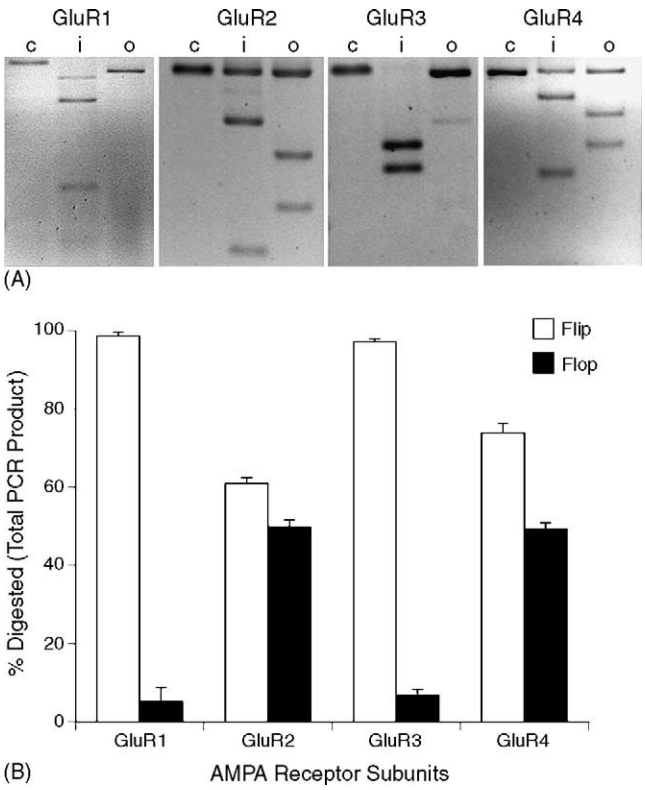

Fig. 2. (A) Examples of splice-variant specific restriction digest of each AMPA receptor subunit PCR product; c: undigested control PCR product; i: flip-specific digest; o: flop-specific digest. (B) Relative proportion of flip (white bars) and flop (black bars) splice variants for each AMPA receptor subunit. Bars indicate mean ± S.E.M. for five rats.

($p = .0005$); GluR2 greater than GluR3 or GluR4 ($p < .00001$); and GluR4 greater than GluR3 ($p = .007$). The student's *t*-test was used to determine the statistical differences in subunit proportions.

In addition to subunit composition, alternative splicing imparts distinct kinetic properties to currents mediated by AMPARs. Therefore, we also determined the relative abundance of flip and flop using methods described in Table 3. Our results demonstrate that mRNAs for both isoforms of each GluR1–4 subunit are expressed in the OB (see Fig. 2A). However, the relative expression of flip versus flop varied among GluR1–4 subunits. GluR2 and GluR4 transcripts were composed of similar amounts of both variants (GluR2: 55 ± 0.8% flip, 45 ± 0.8% flop; GluR4: 60 ± 0.7% flip, 40 ± 0.7% flop). In contrast, the GluR1 and GluR3 transcripts consisted mostly of the flip variant (GluR1: 95.3 ± 3.2% flip, 4.6 ± 3.2% flop; GluR3: 93.5 ± 1.3% flip, 6.5 ± 1.3% flop) (see Fig. 2B). For each subunit, differences in the proportions of flip and flop were statistically significant ($p < .004$), using a one sample *t*-test with a hypothesized mean of 0.5.

AMPARs are present at all identified excitatory synapses in the OB, so their molecular properties likely influence the processing of odor information. This study is the first to quantify the relative abundances of AMPAR subunit and splice variant transcripts in the bulb. Results from RT-PCR analyses demonstrate mRNA expression of each of the AMPAR subunits (GluR1–4), with GluR1 and 2 being the most highly expressed. Our data also indicate that the flip and flop isoforms of each subunit are expressed, further contributing to the molecular diversity of OB AMPARs.

We chose 5-day-old rats for the present study to compliment our previous electrophysiological analyses of AMPARs from animals of a similar age. Although the developmental regulation of OB AMPARs is unknown, previous results from adult animals suggest that GluR1–4 subunits are expressed in the OB in distinct laminar, cellular, and subcellular distributions. In one study, immunoreactivity for GluR4 was heavy in the olfactory nerve layer and external plexiform layer (EPL), while GluR2/3 immunoreactivity was heavy in the granule cell layer and EPL [21]. Whereas mitral/tufted (M/T) cells, the bulb projection neurons, expressed GluR1 and GluR4, granule cells, a type of interneuron, expressed these subunits very weakly or not at all [21]. The use of a combined antibody

(GluR2/3) precluded determination of whether proteins for both GluR2 and GluR3 were expressed. Thus, our quantification of the relative abundance of each subunit represents additional evidence that all AMPAR subunits are expressed in the bulb.

Although mRNAs for all GluR1–4 subunits were present, the relative abundance of GluR2 was highest. Previous results in the hippocampus may help explain this finding. Using immunoprecipitation with subunit-specific antibodies, Wenthold et al. [36] showed that CA1/CA2 pyramidal neurons express two major types of AMPAR complexes: those made up of GluR1 and GluR2 and those made up of GluR2 and GluR3. Few AMPARs contained both GluR1 and GluR3, and approximately 8% were homomeric GluR1 receptors. These results are consistent with the notion that tetrameric complexes of heteromeric receptors contain a maximum of two different subunits [2,20].

Later studies demonstrated the functional significance of these two major populations of AMPARs on hippocampal neurons. Whereas GluR1–R2 receptors are added to the synapse in an activity-dependent manner [29], GluR2–R3 receptors are added constitutively [18,19]. Furthermore, GluR2 appears to play a critical role in controlling the assembly of AMPARs. Using a GluR2 knock-out mouse, Sans et al. [28] showed that, in the absence of GluR2, aberrant receptor complexes composed of GluR1 and GluR3 were formed in the hippocampus and that both homomeric and heteromeric receptors were less efficiently expressed at the synapse. Thus, the high relative abundance of GluR2 in the OB may be important to the formation of functional AMPARs.

Our other significant finding was heterogeneity in the relative abundances of the splice variants. Whereas the GluR2 and GluR4 transcripts consisted of similar amounts of flip and flop, the GluR1 and GluR3 transcripts consisted mostly of flip. In addition to subunit composition, alternative splicing influences the kinetics of AMPAR deactivation and desensitization [17,22,25,31]. For most subunits, the flip form desensitizes more slowly and less profoundly than the flop form [8,22]. Thus, neurons with receptors primarily composed of flip subunits tend to have slower but larger (less desensitized) synaptic currents than neurons with AMPARs primarily composed of flop subunits [10,11].

Flip subunits are also thought to be dominant in determining the behavior of heteromeric AMPARs. Thus, our findings may suggest that the processing of olfactory information requires slower synaptic currents than the processing of other types of sensory information. For example, Ravindranathan et al. [26] found higher relative abundances of flop than flip for GluR1–4 subunits expressed in auditory neurons. They concluded that the high content of GluR3 flop and GluR4 flop in these AMPARs contributes to very rapid synaptic responses, facilitating the high fidelity necessary for auditory processing.

The use of RT-PCR to quantify the relative proportions of GluR subunits and flip/flop variants has several caveats. PCR as employed here does not allow absolute quantification of levels of GluR mRNA expression but rather permits determination of their relative abundance. Furthermore, the measurement of relative proportions assumes that there is equivalent amplification of GluR1–4 (and their isoforms) by the panGluR primers as well as equivalent (or complete) digestion of individual subunits by the subunit-specific restriction enzymes. Ravindranathan et al. [26] explored this latter assumption in a study of auditory neurons. After comparing the relative abundances of GluR1–4 mRNAs determined by RT-PCR with those determined by single-neuron, mRNA profiling, they concluded that both methods produced similar patterns and rank orders of relative abundance of the four subunits.

Because the source of the RNA extracted was homogenized olfactory bulbs, it also was not possible to determine the cellular localization of GluR expression. Our previous electrophysiology results [4], along with prior immunohistochemical data [21], suggest that subunits and splice variants are differentially expressed between neuronal subtypes in the OB. Despite these caveats, the use of RT-PCR with specific primers and restriction enzymes provides greater sensitivity and sequence specificity than can be achieved with other techniques, such as immunohistochemistry or in situ hybridization.

In conclusion, RT-PCR provided a profile of GluR1–4 expression in the OB that is consistent with prior immunohistochemical [21] and electrophysiological [4] evidence of subunit and splice variant diversity. As AMPAR kinetics help determine the timing and efficacy of synaptic transmission [10,13,23], heterogeneity in the relative abundance and/or distribution of flip and flop subunits at excitatory synapses could be important to temporal components of olfactory information processing (e.g., glomerular synchronization, correlated spiking).

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