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# Group I and II metabotropic glutamate receptor expression in cultured rat spinal cord astrocytes

G.A. Silva[a,d], E. Theriault[b,c,d], L.R. Mills[a,d], P.S. Pennefather[a,c,d,e], C.J. Feeney[c,d,*]

[a]Department of Physiology, University of Toronto, 19 Russell Street, Toronto, Ontario, M5S 2S2, Canada
[b]Department of Surgery (Division of Neurosurgery), University of Toronto, 19 Russell Street, Toronto, Ontario, M5S 2S2, Canada
[c]The Institute of Medical Science, University of Toronto, University of Toronto, 19 Russell Street, Toronto, Ontario, M5S 2S2, Canada
[d]The Playfair Neuroscience Unit, The Toronto Hospital Research Institute, Toronto, Ontario, Canada
[e]The Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, Ontario, M5S 2S2, Canada

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**Abstract**

We have examined the development of expression of group I and II metabotropic glutamate receptors (mGluRs) in pure rat spinal cord astrocyte cultures, using immunocytological and calcium imaging techniques. mGluR1$\alpha$ and mGluR2/3 antibodies were found to label roughly 10% of the total astrocyte population at all time points examined, whereas mGluR5 was poorly expressed in our culture system. Results from intracellular $Ca^{2+}$ imaging experiments, measured using fura-2 ratio imaging, suggest that 20% of these cultured astrocytes express functional group I mGluRs (mGluR1 and/or 5). Our results contrast with previously published work in cultured cortical astrocytes where mGluR5 and not mGluR1 is expressed, suggesting that cultured astrocytes from different parts of the CNS exhibit different patterns of mGluR expression. 

*Keywords:* In vitro; Immunocytochemistry; Calcium imaging; G protein-coupled receptors

Metabotropic glutamate receptors (mGluR) are a family of G protein-coupled, seven transmembrane domain receptors that exert a variety of effects on second messenger systems and ion channels [14]. These receptors form a family of at least eight different subtypes (mGluR1–8), with several possible splice variants. Based on agonist pharmacology, coupling to specific signal transduction pathways, and sequence homology, three sub-groupings of mGluRs have been suggested. Group I (mGluR1 and 5) are activated by quisqualate and are coupled to inositol-(1,4,5)-trisphophate ($IP_3$)/$Ca^{2+}$ signal transduction, while group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) are quisqualate insensitive and negatively coupled to adenylate cyclase pathways [14]. Interest in mGluRs has risen with recent reports suggesting that their activation may be involved in both promoting (i.e., group I activation [9]), or protecting against excitotoxic neuronal damage (i.e., group II activation [2])

Glial cells play an important homeostatic role in the regulation of the neuronal environment such as: regulation of neurotransmitter inactivation/re-uptake, formation of the blood-brain-barrier, pH regulation, providing of substrates for energy metabolism, and $K^+$ buffering [16]. It has been suggested that glial (i.e., astrocytic) $Ca^{2+}$ waves could make up an extraneuronal signaling pathway in the CNS [4–6]. Glutamate has been shown to both raise intracellular $Ca^{2+}$, and activate PKC through a G protein/mGluR/$IP_3$ mediated pathway in cultured cortical astrocytes [4,12]. Astrocyte processes are often found in close association with synapses, and are known to possess a variety of neurotransmitter receptors, including mGluRs [7]. Not surprisingly therefore, several reports suggest that intercellular $[Ca^{2+}]_i$ waves in astrocytes can be triggered by neuronal activity

* Corresponding author. Tel.: +1-416-978-6609; fax. +1-416-978-8511; e-mail: chris.feeney@utoronto.ca

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and glutamate release [5,10,13,15]. Thus, an understanding of the characteristics of mGluR expression patterns, and mGluR mediated responses in spinal cord astrocytes should help in defining glutaminergic-based communication and toxicity in this part of the CNS.

We have recently described the expression pattern of mGluRs in the rat spinal cord [18], and have begun to investigate the contribution of group I mGluRs in white matter injury [1]. In order to address these issues at a cellular level, we have developed an in vitro system of pure rat spinal cord astrocytes (SCA [17]), and hereinafter characterize the expression of group I and II mGluRs, and the signaling pathway of group I mGluRs.

Astrocytic cultures were established as previously described [17]. Cervical and lumbar spinal cords from newborn Wistar rat pups were dissected and cleaned of meninges, and dissagregated by means of a 75 $\mu$m pore size Nitex mesh (B and SH Thompson Co.). Culture medium used contained: minimum essential medium with Earle's salts, 10% horse serum, 50 units/ml penicillin/streptomycin (all from Gibco BRL). Cell suspensions were plated onto untreated glass coverslips yielding cultures >99% immunopositive for glial fibrillary acidic protein.

For immunocytochemical analysis, cultures were washed several times in 0.1 M phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde for 10 min at 4°C. Cultures were then washed several times in PBS, and exposed to a blocking solution of 10% normal goat serum (NGS, 10 minutes, 22°C). Primary antibody incubation of polyclonal anti-mGluR1$\alpha$ (Chemicon International), polyclonal anti-mGluR2/3 (Chemicon International), polyclonal anti-mGluR5 (Upstate Biotechnology), and secondary antibody incubations of biotinylated anti-rabbit IgG, or Texas-Red conjugated anti-rabbit secondary antibody (Vector Laboratories), all took place in Tris-buffered 2% NGS (1 h, 22°C). Biotinylated anti-rabbit IgG was detected by means of an avidin-biotin complexed horseradish peroxidase conjugate incubation and subsequent chromogenic treatment (0.05% diaminobenzidine tetrahydrochloride/0.03% $H_2O_2$). Immunolabeled cultures were then air-dried overnight, cleared with xylene, and coverslipped with permanent mounting medium (DPX Mountant, BDH Inc.). Quantitative immunocytological analysis for each mGluR antibody, processed with diaminobenzidine tetrahydrochloride, was independently carried-out on three cultures for each time point (3, 7, 14 and 21 DIV) obtained from a total of four different rat pup liters. Ten fields of astrocytes (at 400 × magnification) were chosen at random from each culture, and the density of immunopositive cells determined.

Cultures utilized in calcium imaging experiments were washed several times in balanced salt solution (BSS; 137 mM NaCl, 5.3 mM KCl, 3 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 25 mM glucose, 6.7 mM Hepes acid, and 3.3 mM Hepes salt, pH 7.4), and loaded with 4 $\mu$M fura-2 acetoxymethyl ester (fura-2 AM, Molecular Probes) dissolved in BSS containing 0.05% bovine serum albumin, for 1 h at 36°C. The cultures were then washed several times in BSS and mounted over an inverted Zeiss AX135 microscope. All chemical reagents (i.e., 1*S*,3*R*-ACPD (t-ACPD), 6-cyano-7-nitroquinoxaline-2,3,-dione (CNQX), D(−)-2-amino-5-phosphonopentanoic acid (D-AP5), all from Tocris Cookson) were delivered in BSS to the cultures by means of a gravity driven perfusion system. Fluorescence >510 nm emitted from these cells after alternating exposures to 340 and 380 nm excitation, was gathered with a highly sensitive CCD camera (Dage CCD-72; Dage Inc) and photointensifier (Videoscope Inc), and digitized using Image 1 software (Universal Imaging Corp). Ratio image acquisition typically took place at 3 s intervals, at 20°C. Fura-2 calibration was achieved by exposing fura-2 AM loaded sister cultures in BSS to the $Ca^{2+}$ ionophore 4-bromo A-23187 (Molecular Probes) to obtain $R_{max}$ (11.7), and then replacing the bathing solution with a $Ca^{2+}$-free BSS supplemented with 100 $\mu$M EGTA, to obtain $R_{min}$ (0.20), and using a $K_d$ for fura-2 of 224 nM. For single cell $[Ca^{2+}]_i$ recordings, random fields of astrocytes, typically including 30–50 fura-2 loaded cells, were chosen.

The pattern of mGluR immunolabeling consisted of a heterogeneous punctate staining of astrocytic cell bodies and processes, with minimal labeling of the nucleus and

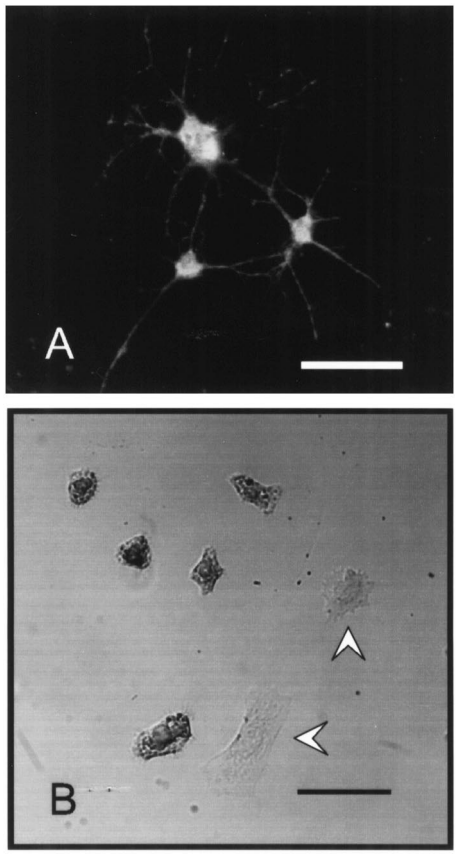


Fig. 1. Photomicrographs of mGluR immunolabeling in cultured rat astrocytes. (A) Fluorescence image of mGluR1$\alpha$ immunopositive stellate astrocytes at 3 DIV. (B) Bright-field image of five mGluR2/3 immunopositive astrocytes alongside two unlabelled astrocytes (arrows). Calibration bar = 100 $\mu$m.

Table 1

Density of mGluR-immunopositive astrocytes in spinal cord astrocyte cultures at 3, 7, 14, and 21 days in vitro (DIV)

| | Cell density ($cm^{-2}$) | | | |
|---|---|---|---|---|
| | 3 DIV | 7 DIV | 14 DIV | 21 DIV |
| All cells in field | $4.0 \times 10^3$ | $4.0 \times 10^3$ | $2.0 \times 10^4$ | $2.0 \times 10^4$ |
| mGluR1$\alpha$ positive | $4.0 \times 10^2$ | $4.5 \times 10^2$ | $1.3 \times 10^3$ | $1.6 \times 10^3$ |
| mGluR5 positive | 0 | 0 | $1.2 \times 10^2$ | $6.0 \times 10^2$ |
| mGluR2/3 positive | $2.8 \times 10^2$ | $3.6 \times 10^2$ | $1.8 \times 10^3$ | $1.4 \times 10^3$ |

its immediate surrounding areas (see Fig. 1). Immunopositive astrocytes were frequently found together, thus forming clusters of mGluR-positive cells separated by regions of mGluR immunonegative astrocytes. However, numerous individual 'isolated' immunopositive astrocytes were also present in these cultures at all time points. The clusters of immunopositive astrocytes appeared to be randomly distributed over the entire culture surface. Table 1 summarizes the density of mGluR immunopositive astrocytes in our SCA cultures. mGluR1$\alpha$ was expressed in roughly 10% of the total astrocyte population at all time points investigated. However mGluR5 was undetectable at 3 and 7 DIV, and expressed in only 0.6% and 3% of the total population of astrocytes at 14 DIV and 21 DIV, respectively. For comparison, group II immunoreactivity (mGluR2/3) was measured and likewise found on approximately 10% SCA at all time points tested. Cultures processed for immunocytochemistry in the absence of primary mGluR antibodies were found to be completely absent of labeling (i.e., no non-specific secondary antibody labeling was observed).

These results parallel in vivo studies [18,19] where mGluR1$\alpha$, 2, 3 and 5 were found to be expressed in neonatal and adult spinal cord. As well, they contrast with published reports of mGluR expression in cortical astrocytes which have been shown to express mGluR5, but not mGluR1$\alpha$ [8,11]. In addition, Condrelli et al. [3] have recently investigated mGluR expression in rat cortical astrocyte cultures, but were not able to demonstrate the presence of mGluR1, 2, 3, 4, and 7 mRNA. Thus our report not only is the first to characterize the expression patterns of group I and II mGluR subtypes on cultured rat spinal cord astrocytes, but also indicates that cultured astrocytes from different parts of the CNS exhibit different patterns of mGluR expression.

To test the functionality of the group I mGluRs present on these cultured SCA, confluent cultures (14–24 DIV) were loaded with the cell permeable, fluorescent calcium indicator fura-2AM, and challenged with the mGluR specific agonist t-ACPD (100 $\mu$M). Single cell recordings of t-ACPD induced increases in $[Ca^{2+}]_i$ were obtained (see Fig. 2A). t-ACPD was found to cause an increase in $[Ca^{2+}]_i$ (greater than 2 fold from resting $[Ca^{2+}]_i$ levels) in 23.0 ± 9.0% (mean ± SEM) at 14–16 DIV ($n$ = 9 cultures), and 20.9 ± 13.5% (mean ± SEM) at 21–24 DIV ($n$ = 10 cultures) of cells. Similar to the immunocytochemistry, t-ACPD induced increases in $[Ca^{2+}]_i$ occurred both in clusters of cells, or in single isolated responding cells. It is clear however that the percentage of cells responding to t-ACPD with a rise in $[Ca^{2+}]_i$ (i.e., those cells which express mGluR1 and/or mGluR5) is higher than our immunocytochemistry results would suggest (see Table 1). One possible explanation for this finding is that these astrocytes may be expressing additional splice variants of group I mGluRs [14] which are not recognized by the antibodies used in the present study.

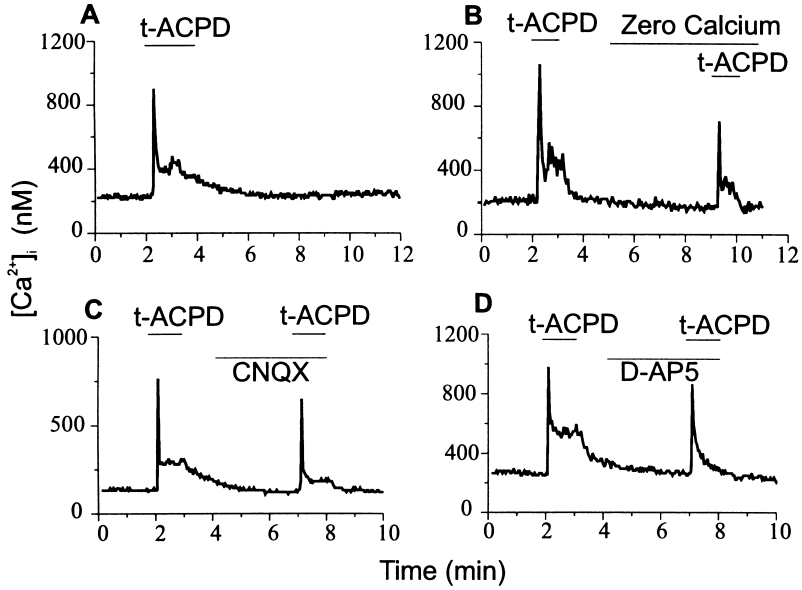


Fig. 2. t-ACPD-induced $[Ca^{2+}]_i$ responses in fura-2AM loaded spinal cord astrocytes. The effects of 100 $\mu$M t-ACPD on $[Ca^{2+}]_i$ in (A) BSS, (B) $Ca^{2+}$-free BSS containing 100 $\mu$M EGTA, (C) BSS containing the AMPA/kainate antagonist CNQX (100 $\mu$M), and (D) BSS containing the NMDA antagonist d-AP5 (100 $\mu$M). The removal of external calcium, and the presence of ionotropic glutamate receptor antagonists had no apparent effect on the t-ACPD responsiveness of these astrocytes. Representative data of responding cells are presented. Total number of cell culture preparations analyzed was 24 (A), 8 (B), 6 (C), 6 (D).

The t-ACPD-induced increase in $[Ca^{2+}]_i$ was found to be either a single spike, or a spike followed by a sustained $[Ca^{2+}]_i$ increase above baseline (see Fig. 2A). It has been previously reported that the activation of mGluR5 leads to an oscillatory $[Ca^{2+}]_i$ response, whereas mGluR1 activation leads to a single-peaked, non-oscillatory $[Ca^{2+}]_i$ response [11]. Oscillatory $[Ca^{2+}]_i$ responses induced by 100 $\mu$M t-ACPD of the type previously described [11] were never observed in our SCA cultures (12–24 DIV, $n = 35$). This is perhaps not surprising, given our immunocytochemical evidence that mGluR5 is expressed on only a small percentage of cultured spinal cord astrocytes.

To further characterize the t-ACPD-induced changes in $[Ca^{2+}]_i$ in cultured SCA, we first examined its dependence on external $Ca^{2+}$ in an EGTA containing, $Ca^{2+}$-free BSS. t-ACPD was found to elicit a significant rise in $[Ca^{2+}]_i$, even in the absence of external $Ca^{2+}$ (see Fig. 2B). To rule out the possibility of ionotropic glutamate receptor (iGluR) involvement in this t-ACPD elicited $Ca^{2+}$ response, cultures were first perfused with either the AMPA/kainate antagonist CNQX or the NMDA antagonist D-AP5 [11], then challenged with 100 $\mu$M t-ACPD. These iGluR antagonists were found to be ineffective in blocking the t-ACPD induced $[Ca^{2+}]_i$ response (see Fig. 2C,D). Thus it appears that t-ACPD can elicit a rise in $[Ca^{2+}]_i$ in cultured SCA via the activation of group I mGluRs (primarily mGluR1), and subsequent mobilization of $Ca^{2+}$ from internal stores.

Differences in patterns of mGluR expression in different regions of the CNS could have broad implications for researcher in multiple fields of study. Those interested in cell–cell communication, as well as those studying the putative neuroprotective or neurotoxic functions of mGluR activation should benefit from a better understanding of the multiple cell types which possess these receptors.

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