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# The viral mimetic polyinosinic:polycytidylic acid (poly I:C) induces cellular responses in primary cultures from rat brain sites with an incomplete blood–brain barrier

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## HIGHLIGHTS

- The TLR3-agonist poly I:C induces $Ca^{2+}$-transients in cultured astrocytes and neurons from OVLT and AP.
- The increase in intracellular $Ca^{2+}$ in activated astrocytes occurs with shorter onset latency compared to neurons.
- Poly I:C-stimulated microcultures from OVLT and AP release bioactive TNF and IL-6 into the supernatant.

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## ABSTRACT

Primary microcultures of the *organum vasculosum laminae terminalis* (OVLT) and the *area postrema* (AP), brain sites with an incomplete blood–brain barrier, were established from topographically excised rat pup tissue, with cellular identification by marker protein-specific immunocytochemistry. Employing the ratio calcium imaging technique, we showed for the first time that polyinosinic:polycytidylic acid (poly I:C) can induce calcium signalling in single OVLT and AP cells. Poly I:C stimulation caused fast, transient rises in intracellular calcium in about 5% of neurons and astrocytes and some microglial cells. Frequently, the responses of astrocytes and microglial cells showed a shorter onset-latency compared to neurons. In addition, exposure to poly I:C led to a time dependent release of bioactive tumour necrosis factor (TNF) and interleukin-6 (IL-6) into the supernatants of OVLT and AP cultures. The demonstration of direct cellular responses of OVLT- and AP-intrinsic cells to stimulations with poly I:C is in agreement with the discovered existence of Toll-like receptor 3 (TLR3), the cognate receptor for poly I:C, in the brain.



## 1. Introduction

The OVLT and the AP belong to the so called sensory circumventricular organs (sCVOs) which lack a tight blood–brain barrier and are composed of glial and neuronal elements. These specialized brain sites can thus act as sensors for circulating chemical messengers [15]. Both brain sites are thought to be involved in the manifestation of brain-controlled unspecific signs of illness including fever and anorexia [2,8,18]. According to the observation that pro-inflammatory cytokines are released under the influence of so-called pathogen-associated molecular patterns (PAMPs) from activated immune cells, a number of cellular and molecular studies have been preformed with the aim to identify cytokine receptors and responses to cytokines in the OVLT and the AP [18]. In addition, direct responses of both brain sites to bacterial LPS have been reported due to the existence of TLR4 and other TLRs in these specialized structures [13,14]. For example, small but consistent populations of OVLT and AP cells responded to LPS with fast transient rises in their intracellular calcium concentration $[Ca^{2+}]_i$ [17,25]. However, the capacity of distinct PAMPs to evoke such responses differs substantially in so far as diacylated lipopeptides (TLR2/6-agonists) as opposed to LPS (TLR4-agonist) failed to induce calcium transients in microcultures from both brain sites [17,25]. TLR3 is the receptor that recognizes double-stranded RNA, such as poly I:C, and it is located in the endosomal compartment of the cell [1]. Poly I:C is used to model viral infections, and there are not only similarities, but also differences between the responses to LPS versus poly I:C in vivo [5,7,12,22,23]. Basal expression of TLR3 in the central nervous system (CNS) has been detected, which is stronger than that noted for other TLRs and is concentrated in astrocytes [6]. We therefore aimed to investigate whether stimulation of OVLT and AP microcultures with the TLR3-agonist poly I:C might result in measurable changes of $[Ca^{2+}]_i$ in cells from both brain sites and to determine the phenotypes of investigated cells

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immunocytochemically. We further tested the capacity of OVLT and AP primary cultures to produce bioactive cytokines in response to exposure with poly I:C.

## 2. Materials and methods

### 2.1. Animals

Wistar rat pups of both sexes obtained from an in-house breeding colony were used for all experiments, with parent animals originating from Charles River WIGA (Sulzfeld, Germany). Animal care, breeding and experimental procedures were conducted according to the guidelines approved by the Hessian Ethical Committee. Room temperature was controlled at 24 ± 1 °C and relative humidity at 50%, and artificial lights were on from 7:00 AM to 7:00 PM.

### 2.2. Preparation and stimulation of OVLT and AP microcultures

Primary microcultures of the rat OVLT and AP were established from topographically excised brain tissue of 5–6 day old Wistar rat pups as previously described [17,25,26]. The dissociated OVLT or AP cells were plated onto pre-warmed, poly-L-lysine (1 mg/ml $H_2O$; Sigma Aldrich) coated CELLocate® glass coverslips (Eppendorf, Hamburg, Germany) forming the bottom of a reusable Flexiperm-micro-12 well (6 mm diameter; Greiner Bio-One GmbH, Solingen, Germany) to ensure sufficient cell density despite limited absolute cell number, and cultured in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. The medium was exchanged the next day to remove cellular debris and thereafter every 2 days during the culture period. The cells were employed for intracellular calcium measurements and a subsequent immunocytochemical characterization; supernatants were used for determination of cytokines.

Synthetic double stranded RNA (poly I:C, Sigma Aldrich) was employed as an agonist for TLR3. The substance was dissolved in phosphate buffered saline (PBS, pH 7.4) at concentrations of 1 mg/ml and added to the perfusion chamber by bolus application to achieve a final concentration of 100 μg/ml during a 1 min arrest of the superfusion pump (minipuls-3; Abimed Analysen-Technik, Langenfeld, Germany). The dose was chosen according to preliminary recordings from pilot studies, in which several doses were tested. A bolus control application with PBS was performed prior to stimulation with poly I:C. At the end of the experiment, cells were exposed to buffer with high calcium concentration (50 mM). This treatment served as vitality test, especially for neurons [17,25,26].

### 2.3. Measurement of intracellular calcium

After 5–6 days cultivation on individual CELLocate® glass coverslips, the cells were loaded with 2 μM fura-2-AM (MoBiTec GmbH, Göttingen, Germany) in complete medium for 45 min in a humidified atmosphere of 5% $CO_2$/95% air at 37 °C. For intracellular $Ca^{2+}$-measurements, the coverslips were placed under an inverted microscope (IMT-2; Olympus Optical, Hamburg, Germany) in a specially constructed Teflon® culture chamber and superfused with buffer [17,25]. Fluorescence measurements were performed using a filterwheel-based excitation system and analyzed with MetaFluor 4.5 software (Visitron, Puchheim, Germany). After defining regions of interest for single cells by a continuously variable aperture, the time course of emitted fluorescence (>515 nm) after alternating excitations at 340 and 380 nm, respectively, was recorded at 0.2 Hz using a Visicam 12 BIT digital CCD-camera (Visitron). The 340/380 ratios proportional to $[Ca^{2+}]_i$ were computed and analyzed [9,17,25,26]. Phenotypic identification of cultured neurons and glial cells was confirmed by immuno-labelling with polyclonal antisera or monoclonal antibodies directed against cell-specific marker proteins as described previously [17,25,26].

### 2.4. Cytokine measurements

After 5–6 days cultivation on CELLocate® glass coverslips, the OVLT or AP cells were incubated with poly I:C (100 μg/ml) or solvent in complete medium for 0, 15, 30, 60, 90 or 120 min in a humidified atmosphere of 5% $CO_2$/95% air at 37 °C. The supernatants were removed from the cells for later measurements of cytokines. Determination of TNF-α was performed by a bioassay based on the cytotoxic effect of TNF-α on the mouse fibrosarcoma cell line WEHI 164 subclone 13 and a murine TNF-α standard (code 88/532; National Institute for Biological Standards and Control, South Mimms, UK [17]). Measurement of IL-6 was performed by a bioassay based on a dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line and a human IL-6 standard (code 89/548; National Institute of Standards and Control, South Mimms, UK; [17]). The levels TNF-α and IL-6, which we determined in our samples, do not reflect absolute cytokine concentrations but rather bioactivity in relation to established international standards. To confirm the specificity of the assays, we added 1 μg of a neutralizing TNF binding protein (TNFbp), a synthetic form of the type 1 soluble TNF-receptor [20] or 10 μl of a neutralizing antiserum against rat-IL-6 [10] to the supernatants of some poly I:C-stimulated microcultures. The presence of TNFbp or IL-6-antiserum abrogated TNF- or IL-6-bioactivity in these samples completely (TNF) or almost completely (IL-6).

### 2.5. Evaluation and statistics

The numbers of poly I:C responsive cells of a given cellular phenotype, under the experimental conditions defined above, were expressed as a percentage (%) of all cells of this phenotype investigated. Stimulus-induced transient increases of $[Ca^{2+}]_i$ were expressed as the difference between resting $[Ca^{2+}]_i$ measured prior to the respective stimulation and the stimulus-induced $[Ca^{2+}]_i$ peak (Δratio [340/380 nm]). A difference in Δratio [340/380 nm] of more than 0.1 was considered to be a stimulus-induced $Ca^{2+}$-signal, which can be regarded as a strict exclusion criterion. Values of Δratios and cytokine levels are presented as means ± standard error of the mean (SEM) and compared by an analysis of variance (ANOVA) followed by Scheffe's post hoc test (Abacus Concepts, Berkeley, CA, U.S.A.).

## 3. Results

### 3.1. Poly I:C induces calcium signals in cells of OVLT and AP microcultures

After 5 days of in vitro differentiation primary rat OVLT and AP microcultures contained small-sized bi- or tripolar neurons expressing microtubule-associated protein 2a + 2b, squamous or stellate astrocytes richly endowed with cytoskeletal glial fibrillary acidic protein, mature oligodendrocytes expressing 2′3′-cyclic nucleotide 3′-phosphoesterase (CNPase) and quiescent as well as some activated microglial cells staining for CD68 (synonym: ED-1) as previously described [17,25]. Endothelial cells and fibroblasts proved not to be present as indicated by negative immunolabelling for fibronectin and von Willebrand factor. Cells of all phenotypes were subjected to intracellular calcium imaging with poly I:C as putative agonist for TLR3. An example for calcium traces of a group of four OVLT cells, two neurons and two astrocytes positioned in rather close vicinity on the glass coverslip, are shown in Fig. 1.

In two out of four investigated cells a bolus application of poly I:C induced a transient increase of $[Ca^{2+}]_i$, while a bolus injection of