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# 2 Distinct P2Y receptors mediate extension and 3 retraction of microglial processes in epileptic 4 and peri-tumoral human tissue

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22 Short title: Purines and motility of human microglia.

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## 33 ABSTRACT

34 Microglia exhibit multiple, phenotype-dependent motility patterns often triggered by purinergic  
35 stimuli. However little data exists on motility of human microglia in pathological situations.

36 Here we examine motility of microglia stained with a fluorescent lectin in tissue slices from female  
37 and male epileptic patients: mesial temporal lobe epilepsy (MTLE) or cortex surrounding glioma  
38 (PTC). Microglial shape varied from ramified to amoeboid cells predominantly in regions of high  
39 neuronal loss or closer to a tumor.

40 Live imaging revealed unstimulated or purine induced microglial motilities including surveillance  
41 movements, membrane ruffling and process extension or retraction.

42 At different concentrations, ADP triggered opposing motilities. Low doses triggered process  
43 extension. It was suppressed by P2Y12 receptor antagonists which also reduced process length and  
44 surveillance movements. Higher purine doses caused process retraction and membrane ruffling,  
45 which were blocked by joint application of P2Y1 and P2Y13 receptor antagonists.

46 Purinergic effects on motility were similar for all microglia tested. Both amoeboid and ramified cells  
47 from MTLE or PTC tissue, expressed P2Y12 receptors. A minority of microglia expressed the  
48 adenosine A2A receptor which has been linked with process withdrawal of rodent cells.

49 Laser-mediated tissue damage let us test the functional significance of these effects. Moderate  
50 damage induced microglial process extension which was blocked by P2Y12 receptor antagonists.

51 Overall, the purine-induced motility of human microglia in epileptic tissue is similar to that of rodent  
52 microglia in that the P2Y12 receptor initiates process extension. It differs in that retraction is  
53 triggered by joint activation of P2Y1/P2Y13 receptors.

54 **KEY WORDS.** live imaging, human microglia, purine, process extension, process retraction.

## 55 SIGNIFICANCE STATEMENT

56 Microglial cells are brain-resident immune cells with multiple functions in healthy or diseased brains.  
57 These diverse functions are associated with distinct phenotypes including different microglial shapes.  
58 In the rodent, purinergic signaling is associated with changes in cell shape such as process extension  
59 towards tissue damage. However there is little data on living human microglia especially in diseased  
60 states. We developing a reliable technique to stain microglia from epileptic and glioma patients to  
61 examine responses to purines. Low intensity purinergic stimuli induced process extension, as in  
62 rodents. In contrast, high intensity stimuli triggered a process withdrawal mediated by both P2Y1 and  
63 P2Y13 receptors. P2Y1/P2Y13 receptor activation has not previously been linked to microglial  
64 morphological changes.

## 65 INTRODUCTION

66 Microglia are brain-resident immune cells with multiple functional phenotypes. In healthy tissue,  
67 ramified microglial processes move constantly to surveil the space around them (Dailey & Waite,  
68 1999; Davalos et al., 2005; Nimmerjahn, Kirchhoff & Helmchen, 2005). During development,  
69 microglia remove superfluous synapses and cells to shape neuronal connectivity (Paolicelli et al.,  
70 2011; Schafer et al., 2012). In response to damage, microglial processes extend toward a site of  
71 injury and phagocytose cellular debris (Koizumi et al., 2007). In many pathologies, microglia liberate  
72 inflammatory mediators (Sanz & Di Virgilio, 2000; Bianco et al., 2005), but they also engage in repair  
73 and resolution (Raposo & Schwartz, 2014).

74 Distinct microglial phenotypes (Streit, Graeber & Kreutzberg, 1988) depend in part on signaling  
75 mediated by purinergic receptors (Hanisch & Kettenmann, 2007). Purines are released depending on  
76 neuronal activity and cell damage (Dale & Frenguelli, 2009). They act on ionotropic P2X receptors  
77 (Khakh & North, 2012), on G-protein coupled P2Y receptors (von Kügelgen, 2006) and on adenosine  
78 receptors expressed by microglia (Chen, Lee & Chern, 2014). Distinct microglial phenotypes have  
79 been linked with activation of different purinergic receptors. Extension of processes towards an  
80 injury is mediated via P2Y12 receptors (Honda et al., 2001; Haynes et al., 2006). P2Y6 receptors  
81 trigger phagocytosis (Koizumi et al., 2007) and P2X7 receptors activate inflammasome processing of  
82 cytokines for secretion (Pelegrin et al., 2008).

83 Responses to purinergic stimuli differ for microglia in an inflammatory context and those in a healthy  
84 brain. While healthy cells extend processes towards a purine source or cell damage (Davalos et al.,  
85 2005; Haynes et al., 2006) processes of microglia in an inflamed brain retract. This difference has  
86 been attributed to a switch in receptor expression from P2Y12 to adenosine A2A receptors (Orr et al.,  
87 2009; Gyoneva et al., 2014). However motility patterns induced by purinergic stimulation have rarely  
88 been studied for human microglia in a pathological or inflammatory context.

89 We therefore examined the effects of purines on motility in microglia of tissue from patients with  
90 medial temporal lobe epilepsies and with epileptic cortical tissue surrounding or infiltrated by  
91 gliomas. A fluorescent lectin (Bordey & Spencer, 2003; Schwendele et al., 2012) was used to label  
92 microglia, enabling 2-photon imaging of living cells over several hours. In the absence of stimulation,  
93 microglia varied from round cells, through microglia with few processes to highly ramified cells  
94 (Boche, Perry & Nicoll, 2013; Morin-Brureau et al., 2018). We found purinergic stimuli induced  
95 distinct and opposing motility responses: extension at low doses and retraction of processes at  
96 higher doses. Our data suggest that extension was dependent on activation of P2Y12 receptors as in

97 the rodent. However process retraction was not mediated via A2A receptors (Orr et al., 2009), but  
98 rather by joint activation of P2Y1 and P2Y13 receptors.

99

## 100 MATERIALS AND METHODS

### 101 *Tissue from epileptic patients*

102 We compared microglia in tissue from patients diagnosed with two syndromes. Temporal lobe tissue  
103 was obtained after operations (CHU Pitié-Salpêtrière, Dr B Mathon, Dr S Clemenceau) on patients  
104 diagnosed with pharmaco-resistant medial temporal lobe epilepsies associated with a hippocampal  
105 sclerosis (MLTE; Le Duigou et al., 2018; Morin-Brureau et al., 2018). Tissue was obtained from 8 MTLE  
106 patients (age 29-64, 4 women, 4 men, left or right temporal lobe sclerosis). Epileptic activities were  
107 evident in surface EEG records and hippocampal sclerosis was confirmed by non-invasive imaging.  
108 Cortical tissue was obtained from peri-tumoral regions (PTC) in patients diagnosed with cortical brain  
109 tumors (CHU St Anne, Dr J Pallud; CHU Pitié-Salpetrière, Dr L Cappelle; Pallud et al., 2014). Cortical  
110 tissue was obtained from 17 patients (age 25-63; 5 women, 12 men; 4 diagnosed with astrocytoma, 7  
111 with glioblastoma, 7 with oligodendro-glioma; WHO grades I-IV). 7 of these 17 patients exhibited  
112 seizures before surgery. Epileptic activities may have been missed in surface EEG records from the  
113 other patients since sites of seizure generation are often restricted and /or deep in the brain.  
114 Minimal cauterization during surgery enhanced the quality of tissue slices. Patients all gave a written,  
115 informed consent. Protocols were approved by the Comité de protection des personnes, Ile de  
116 France 1 (C16-16, 20152482) and followed guidelines of the Comité Consultatif National d’Ethique.

### 117 *Imaging of microglia in human tissue slices*

118 Tissue handling from the operating room to the laboratory was described in Le Duigou et al. (2018).  
119 In brief, tissue was transported in a solution of: sucrose, 248; NaHCO<sub>3</sub>, 26; KCl, 1; CaCl<sub>2</sub>, 1; MgCl<sub>2</sub>, 10;  
120 d-glucose, 10 mM equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, at 2–10 °C. Slices of thickness 300 µm were  
121 cut in the same solution in sterile conditions with a vibrating tissue slicer (HM650V, Microm).

122 We tested two fluorophore-coupled lectins to stain microglia in acute human tissue slices for 2-  
123 photon imaging. The B4 isolectin from Griffonia simplicifolia (GSA I-B4) labels fixed (Boya et al., 1991)  
124 and living microglia (Petersen & Dailey, 2004) as does the tomato lectin from Lycopersion  
125 esculentum (Acarin et al., 1994; Bordey & Spencer, 2003). Staining was not detected further than  
126 ~50 µm from the slice surface, when tomato lectin conjugated to Dy-light 594 (Vector labs) or B4  
127 isolectin conjugated to Alexa Fluor 488 (Thermofisher) were bath applied. We therefore injected

128 lectins from patch pipettes inserted into middle regions of slices, at > 100  $\mu\text{m}$  from the surface.  
129 Lectins were dissolved at 50  $\mu\text{M}$  in a solution of NaCl, 150; KCl, 2.5 and HEPES 10 mM (pH 7.4, no  
130 added  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ ) and ejected by gentle positive pressure applied for  $\sim 1$  min.  
131 Both the tomato lectin and B4 isolectin stained epithelial and endothelial cells of brain blood vessels  
132 (Fig. 1A). However only the tomato lectin provided useful staining of human microglia (Schwendele  
133 et al., 2012). This staining colocalized with Iba1 immunopositivity (Fig. 1B). Fluorescent microglia  
134 were resolved at 5-10 min after injection and strong signals were maintained during observations of  
135 duration up to 2-3 hrs. Possibly the tomato lectin also stains infiltrated peripheral monocytes and  
136 macrophages (Ravizza et al., 2008; Varvel et al., 2016). If so, lectin-positive elements, including cells  
137 associated with blood vessels, might be described as 'tomato lectin positive cells' (see Discussion).  
138 We also used a fluorescent purine (EDA-ADP - ATTO-488, Jena Bioscience) to examine the clearance  
139 of purines from slices. It was dissolved in Tris-HCl (1 mM, pH 7.5) and the time course of the loss of  
140 fluorescence was compared after ejection into a tissue slice or into bath solution.

141 (Figure 1: Tomato lectin vs B4-isolectin. Microglial form)

#### 142 ***2-photon imaging of microglia.***

143 Fluorescent microglia were monitored with a Zeiss Axio Examiner Z1 microscope (Zeiss) using two-  
144 photon illumination (3i Intelligent Imaging) from a Chameleon Ultra II Ti-sapphire laser (Coherent).  
145 Slices of human tissue were placed submerged in a chamber, perfused with a solution of: NaCl, 125;  
146  $\text{NaHCO}_3$ , 26; KCl, 3;  $\text{CaCl}_2$ , 2;  $\text{MgCl}_2$ , 2 and glucose, 10 mM, equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  and  
147 heated to 30-32  $^\circ\text{C}$ .

148 The Dy-light 594 coupled tomato lectin was excited at 800 nm and fluorescence detected by a photo-  
149 multiplier tube with a 616/69 emission filter and a 580 nm dichroic mirror. IB4 isolectin was excited  
150 at 920nm and fluorescence detected after a 525/40 emission filter and a 580nm dichroic mirror.  
151 Water immersion objectives of either 20x, NA 1.0 or 40x NA 1.0 (Zeiss) were used. Microglia were  
152 monitored after a delay of at least 90 min from slice preparation. Optical sections were collected at z-  
153 intervals 1-2  $\mu\text{m}$  over a depth of 50-70  $\mu\text{m}$  in the middle of slices. Complete z-stacks could be  
154 acquired at intervals of 30-60 sec. Images are shown as 2D projections of maximal intensity derived  
155 from z-stacks.

156 In some experiments laser stimuli were used to induce tissue damage. 10-20 pulses (duration 3 s,  
157 wavelength 720nm, power 400-500 mW) were delivered. Bright-field observations suggested  
158 damage was maximal in a zone of diameter  $\sim 10$ -15  $\mu\text{m}$  at the targeted region.

159

160 ***Image analysis and quantification of microglial shape and motility.***

161 Changes in microglial form were measured from sequentially obtained z-stacks with Slidebook  
162 software ((3i Intelligent Imaging). We attempted to minimize two possible sources of artefact. First,  
163 as microglial processes could overlap with those of lectin-stained blood vessels (Fig. 1C), or with  
164 those of other microglia during extension, we used minimal volumes of injected lectin to label few  
165 microglia. Second, since extending processes could extend beyond previously defined volumes,  
166 regions of interest were set to allow for process expansion.

167 The shape of identified, non-overlapping microglia was represented by a mask created on z-stack  
168 derived images. Median filtering and deconvolution with a 'nearest-neighbour' protocol (Slidebook)  
169 reduced noise and improved the resolution of fluorescent cells. Masks were created using a 'segment  
170 mask' tool, defined by Otsu thresholding (Slidebook). The 'refine object' tool was used for manual  
171 correction of threshold or shape, if needed. Finally the 'mask statistics' tool was used to follow the  
172 time course of changes in microglial cross sectional area ( $\mu\text{m}^2$ ) derived from maximal 2-D projections  
173 from z-stacks. Cell volume was derived from complete 3-dimensional reconstructions of  
174 unstimulated single cells or maximally extended or retracted states after purinergic stimuli.

175 Ruffling, continuous extrusion and retraction of microglial surface membrane (Bianco et al., 2005),  
176 was quantified by subtracting sequential 2D maximal projections at defined time intervals. Color-  
177 coding of membrane lost or gained between sequential time points was used to illustrate process  
178 extension and retraction.

179

180 ***Purinergic stimulation of microglia: agonists and antagonists.***

181 We examined microglial mobility and motility responses to bath application of purinergic agonists  
182 and antagonists. Slice preparation might alter the form and motility of human microglia. To reduce  
183 such possible artefacts, the effects of purines were not tested until at least 2 hr after slices were  
184 prepared. Response latencies were measured from the time of switching to the purine containing  
185 solution. Latencies include a delay of ~60 s for new solution to reach sites within a slice. Effects of  
186 purines on microglial processes were measured at a latency of 20 m after switching between  
187 solutions, unless noted otherwise.

188 Initial data was obtained with ADP (10  $\mu\text{M}$  – 2 mM) and ATP (10  $\mu\text{M}$  – 2 mM). Since purines are  
189 hydrolyzed by intrinsic tissue ecto-nucleotidases (from ATP to ADP to adenosine, Yegutkin, 2014), we  
190 also examined responses to a non-hydrolyzable ADP analog, 2MeSADP (2- methylthioadenosine  
191 diphosphate trisodium; 0.001-2  $\mu\text{M}$ ), a non-hydrolyzable ATP analog, 2MeSATP (2-

192 methylthioadenosine triphosphate tetra sodium, 0.1-100  $\mu$ M) and the stable adenosine analog, 2-  
193 chloro-adenosine (10-100  $\mu$ M). 2MeSADP is an agonist at P2Y1, P2Y12 and P2Y13 receptors.  
194 2MeSATP is an agonist at P2Y1, P2Y6 and P2Y13 as well as P2X receptors. 2-chloro-adenosine is an  
195 agonist at A1, A2A, A2B and receptors (Abbracchio et al., 2019).

196 We examined effects on these responses of specific antagonists at distinct purinergic and adrenergic  
197 receptors. As P2Y12 receptor antagonists we used PSB0739 (Tocris 0.1-5  $\mu$ M; Baqi et al., 2009) and  
198 Ticagrelor (Cayman 1-10  $\mu$ M; Cattaneo, 2010). MRS2279 (Tocris 1-10  $\mu$ M; Shinozaki et al., 2014) and  
199 MRS 2500 (Tocris 1-10  $\mu$ M; Quintas et al 2018) were used as antagonists at P2Y1 receptors. MRS2211  
200 (Tocris 5-30  $\mu$ M; Kim et al., 2005) was used as a P2Y13 antagonist. As A2A receptor antagonists we  
201 used Preladenant (Sigma 5  $\mu$ M; Neustadt et al., 2009) and SCH-58261 (5  $\mu$ M; Orr et al., 2009). MRS  
202 2578 (Tocris 10  $\mu$ M) was used as P2Y6 antagonist (Riegel et al., 2011).

203

#### 204 ***Immunostaining and imaging.***

205 Tissue slices (200-300  $\mu$ m) were fixed for immunostaining by immersion for 2 min in PFA (4%  
206 phosphate-buffered paraformaldehyde) heated to 80°C. High temperatures enhance diffusion and  
207 accelerate fixation in slices (Dissing-Olesen & MacVicar, 2015). Immuno-staining was performed on  
208 fixed slices (200-300  $\mu$ m) washed in 0.1 M PBS (phosphate buffered saline) containing 20% DMSO (di-  
209 methyl sulfoxide) and 2% Triton x100. Slices were blocked in 3% BSA (24 hrs; bovine serum albumen)  
210 and then incubated with a primary antibody together with 1% BSA for 7 days at 4°C.

211 We used primary antibodies directed against: Iba1 (Abcam, ab5076; 1/500), P2Y12 (Novus, NBP2-  
212 33870; 1/200) and A2AR (Santa Cruz, sc-32261; 1/100), P2Y1 (Abcam, ab168918; 1/200) and P2Y13  
213 (LSBio, LS- A1622; 1/200). Slices were incubated for 6 days with secondary antibodies: IgG  
214 conjugated with Alexa 488, 555 or 647. These long exposures to primary and secondary antibodies  
215 should enhance penetration throughout fixed slices (Dissing-Olesen & MacVicar, 2015). After  
216 exposure slices were mounted in Fluorosave (Merck) on microscope slides with a cover glass  
217 elevated by 300  $\mu$ m (Fisher Scientific).

218 Structured images were made with an Olympus IX81 fluorescent microscope equipped with filters for  
219 4 emitted light wavelengths and visualized with Retiga EXI camera (Qimaging). Stacks of 10-30 images  
220 at z interval 0.5-1.0  $\mu$ m were acquired with oil immersion objectives (40x, NA 1.3 or 60x, NA 0.9).

221

222

223 **Experimental design and statistical analysis**

224 Data are presented as mean  $\pm$  SEM. Statistical analysis was done and graphs produced using Origin  
225 Pro 2016 software. The significance of differences between groups was assessed with the unpaired,  
226 two-sided Student's t-test, with  $p \leq 0.05$  considered significant. Differences within subjects were  
227 assessed with the paired, two-sided Student's t-test with  $p \leq 0.05$  considered significant.

228

229

## 230 RESULTS

231

### 232 **Lectin labelled microglia in tissue from patients with mesial temporal lobe epilepsy (MTLE) and** 233 **peri-tumoral cortex (PTC).**

234 Tomato lectin labelled microglia in human MTLE and PTC tissue were heterogeneous in form, with  
235 features that were consistent between tissues. Fig. 1C shows examples of cells from PTC tissue and  
236 from the dentate, CA1 region and subiculum of MTLE-HS tissue. Three-dimensional reconstructions  
237 of these cells are shown in Fig. 1D and distributions of cross sectional area for cells from each region  
238 (n=21-58) are plotted in Fig. 1E.

239 Cells of the subiculum typically possessed multiple ramified processes similar to surveilling microglia  
240 of the rodent (Hanisch & Kettenman 2007). In contrast, microglia from the CA1 region where few  
241 neurons survived (Blümcke et al., 2013), were often smaller with fewer, shorter processes or no  
242 processes. Rodent cells with these shapes have been termed 'activated' and 'reactive' microglia  
243 (Streit, Graeber & Kreutzberg, 1988; Davis, Foster & Thomas, 1994). Initial work on human epileptic  
244 tissue identified many 'reactive' microglia (Beach et al., 1995). Microglia of peri-tumoral cortex  
245 exhibited a similar range of shapes. The density of tomato lectin positive cells with few processes was  
246 higher near the tumor (Buckingham & Robel, 2013). The cross-sectional area of cells with no  
247 processes, in MTLE and PTC tissue, was typically 70-180  $\mu\text{m}^2$ . We will not differentiate between  
248 'reactive' and 'activated' microglia, but rather class cells with cross-sectional areas < 300  $\mu\text{m}^2$  (red  
249 lines in Fig. 1E) and with two or less processes as amoeboid cells, and those with areas > 300  $\mu\text{m}^2$  and  
250 more than two processes as ramified (Buckingham & Robel, 2013; Morin-Brureau et al., 2018).

251

### 252 **Responses of microglia to purinergic stimulation**

253 We monitored fluorescent microglia in tissue slices from patients diagnosed with mesial temporal  
254 lobe epilepsies (MTLE) and in peritumoral slices from patients with cortical gliomas (PTC). In our  
255 previous work ~95% of MTLE tissues generate spontaneous interictal-like epileptic activity (Huberfeld  
256 et al., 2011) while such activity is recorded from ~70% of PTC tissue slices (Pallud et al. 2014).

257 Amoeboid microglia of MTLE or PTC tissue moved little in control conditions. Processes of ramified  
258 microglia of MTLE or PTC extended and retracted constantly while their somata moved little.

259 Different doses of ADP or ATP, applied by bath, induced opposing effects on microglial motility. Low  
260 doses (1-10  $\mu\text{M}$  for 15-30 min) induced microglial process extension (Fig. 2A). Processes typically  
261 extended towards the surface of the slice and terminated in bulb-like endings (Fig. 2B). In amoeboid

262 cells application of 10  $\mu$ M ADP (Fig. 2C; **Movie 1**) induced a significant increase of  $80\pm 18\%$  in cross-  
263 sectional area ( $p=0.004$ ;  $t=-4.37$ ;  $n=7$ , 3 MTLE and 4 PTC cells; Fig. 2E) transforming them into  
264 ramified cells. New processes appeared at a latency of 2-8 min (mean  $5.0\pm 1.2$  min; Fig. 2G). The  
265 mean volume of amoeboid cells increased significantly from  $296.5\pm 34.5\ \mu\text{m}^3$  to  $555.8\pm 106.9\ \mu\text{m}^3$  at  
266 full extension (1 MTLE and 4 PTC cells;  $p=0.03$ ;  $t=-3.23$ ).

267 In ramified cells 10  $\mu$ M ADP (Fig. 2D) also significantly increased mean cross-sectional area by  $39\pm 9\%$   
268 ( $p=0.01$ ;  $t=-3.42$ ; 3 MTLE and 4 PTC cells; Fig. 2F). Process extension was initiated at a latency of 6-12  
269 min (mean  $9.4\pm 1.4$  min;  $n=3$  MTLE and 4 PTC microglia). The latency to a 20% increase in cross-  
270 sectional area was consistently greater for ramified than for amoeboid microglia ( $p=0.009$ ;  $t=3.2$ ; Fig.  
271 2G). The mean volume of ramified cells increased significantly from a control value of  $709.2\pm 74.3$   
272  $\mu\text{m}^3$  to  $900.2\pm 30.4\ \mu\text{m}^3$  at full extension ( $p=0.03$ ;  $t=-3.17$ ;  $n=5$ , 1 MTLE and 4 PTC cells; not shown).

273

274 (Figure 2: low dose purinergic responses image from side. Bulb-ending close-up)

275 (Movie 1: process extension of an amoeboid cell induced by low-dose ADP)

276

277 Thus low doses of ADP induced process extension for both initially amoeboid and ramified microglia.  
278 Percentage increases in cross-sectional area and absolute increases in microglial volume were larger  
279 for amoeboid cells. Similar responses were induced by 10  $\mu$ M ATP ( $n=5$ , 2 MTLE and 3 PTC cells not  
280 shown).

281 Higher doses of ADP or ATP (1-2 mM for 30 min) triggered distinct changes in microglial motility.  
282 Amoeboid cells exhibited ruffling (Fig. 3A; **Movie 2**), continuous advances and retractions often of  
283 perisomatic membrane (Bianco et al 2005), but 2 mM ADP induced only minor changes (mean  
284  $7\pm 12\%$ ) in their cross-sectional area ( $p=0.6$ ;  $t=-0.56$ ; 3 MTLE cells, 4 PTC cells; Fig. 3E). Processes of  
285 ramified cells ceased surveillance movements in response to 2mM ADP. They then retracted with a  
286 latency of 5-10 min (mean  $3.4\pm 1.1$  min; Fig. 3C; **Movie 3**). The cross-sectional area of ramified cells  
287 was reduced by  $18\pm 2\%$  ( $p < 0.001$ ;  $t=6.04$ ; 5 MTLE cells and 4 PTC cells; Fig. 3F). The volume of  
288 initially ramified cells was reduced significantly from  $721.6\pm 96.5\ \mu\text{m}^3$  to  $573.7\pm 92.2\ \mu\text{m}^3$  ( $p < 0.001$ ,  
289  $t=6.52$ ; 5 MTLE and 2 PTC cells). Processes of initially ramified microglia did not retract so far as to  
290 convert them into amoeboid cells. Membrane ruffling occurred in ramified cells when retraction was  
291 complete. Cells tested with high dose ADP were not the same as those tested with low-dose ADP.

292 (Figure 3: high dose purinergic responses. Off response. Ruffling)

293 (Movie 2: membrane ruffling of an amoeboid microglia induced by 2 mM ADP)

294 (Movie 3: process retraction of a ramified microglia induced by 2 mM ADP)

295 Unexpectedly process of both ramified and amoeboid microglia extended on switching from 2 mM  
296 ADP back to control ACSF (Fig. 3B, D). This rebound extension consisted of a significant increase  
297 ( $120 \pm 23$  %) in cross-sectional area of amoeboid cells ( $p < 0.001$ ;  $t = -6.40$ , 3 MTLE cells, 4 PTC cells; Fig.  
298 3E; Movie 2). This extension is comparable to that induced by 1-10  $\mu$ M ADP. Rebound process  
299 extension occurred for all ramified microglia on switching to control ACSF (5 MTLE cells and 4 PTC  
300 cells; Fig. 3F; Movie 3). The mean increase in cross-sectional area at after ADP withdrawal was  $31 \pm 5$   
301 % ( $p < 0.001$ ;  $t = -5.44$ ). As for process extensions induced by low purine doses, the latency to rebound  
302 extension was consistently greater for ramified than for amoeboid microglia ( $p = 0.002$ ;  $t = 3.86$ ; Fig.  
303 3G).

304 A similar process extension was induced on switching from 2 mM ADP to 10-50  $\mu$ M ADP ( $n = 4$  cells  
305 not shown). This tends to suggest that the extension may have resulted from exposure to low levels  
306 of ADP during slow clearance from slices. We examined this possibility by comparing the decay of  
307 signals from a fluorescent purine (EDA-ADP - ATTO-488, 1mM) ejected from a patch pipette in the  
308 bath or in middle regions of a tissue slice. Signals decayed in 2-3 sec after ejection into the bath. In  
309 contrast, they decayed more slowly with a double exponential time course when ejection was made  
310 into the slice. Fluorescence levels at 20 min remained at 1.5-2 % of peak values. (Fig 3-1).

311 (Fig 3-1: clearance of a fluorescent purine from slices)

312 Intermediate purine doses (100-500  $\mu$ M ADP, 15-30 min) could initiate either process retraction or  
313 extension in different neighboring cells. Fig. 2-1 plots dose/motility relations as percentage changes  
314 in cross-sectional area for 15 amoeboid and 16 ramified cells (1  $\mu$ M to 2 mM ADP). Maximal mean  
315 increases in cross-sectional area ( $63 \pm 15$  %;  $p = 0.002$ ,  $t = -4.1$ ) occurred in response to 10  $\mu$ M ADP.  
316 Maximal reductions ( $-16 \pm 5$  %;  $p = 0.009$ ;  $t = 3.10$ ) were induced by 2 mM ADP. Process extension when  
317 purinergic stimulation ceased was most strong for 2 mM ADP. Fig. 2-1, C-E plots absolute changes in  
318 cross-sectional area for ramified and amoeboid cells showing a similar switch between process  
319 extension for low doses and retraction for high purine doses.

320 (Fig. 2-1: changes in cross-sectional area for ADP doses of 2  $\mu$ M–2 mM)

321 Overall these data show low and high doses of purines induce two distinct responses, microglial  
322 process extension and retraction comparable for ramified or amoeboid cells (Fig. 2-1,C-E).  
323 Furthermore microglia of MTLE and PTC tissue tended to behave in the same way (Fig 2-2). Mean  
324 process extension induced by 10  $\mu$ M ADP was  $51 \pm 12$  % in 4 ramified PTC cells and  $42 \pm 3$  % in 3  
325 ramified MTLE cells ( $p = 0.48$ ;  $t = 0.75$ ; not significantly different; Fig 2-2, A). Process extension induced  
326 by low dose ADP was  $92 \pm 27$ % for 4 amoeboid PTC cells and  $67 \pm 14$ % for 3 amoeboid cells from MTLE

327 tissue ( $p=0.61$ ;  $t=0.53$ ; not significantly different; Fig 2-2, B). Process retraction induced by 2 mM ADP  
328 was  $18\pm 4\%$  for 4 ramified PTC cells and  $13\pm 3\%$  for 5 ramified MTLE cells ( $p=0.72$ ;  $t= -0,35$ ; not  
329 significantly different; Fig 2-2, C). With these similarities, cells from PTC and MTLE tissues will be  
330 considered together in following data.

331 (Fig 2-2: ADP effects on ramified and amoeboid microglia of PTC and MTLE tissue)

### 332 Evidence for receptor expression.

333 These opposing microglial motilities may depend on distinct receptors. In rodents, activation of the  
334 P2Y<sub>12</sub> receptor initiates microglial process extension (Haynes et al., 2006). Reciprocally, purinergic  
335 stimuli induce process retraction by microglia in an inflammatory context (Gyoneva et al., 2014) via  
336 up-regulated A<sub>2A</sub> receptors (Orr et al., 2009).

337 Tissue from epilepsy patients is in an inflammatory state and some microglia have an amoeboid  
338 shape (Beach et al., 1995; Buckingham & Robel, 2013; Morin-Brureau et al., 2018). We therefore  
339 asked whether amoeboid or ramified cells of MTLE and PTC tissue express P2Y<sub>12</sub> and A<sub>2A</sub> receptors  
340 by immunostaining with specific antibodies (Fig. 4). All Iba1<sup>+</sup> cells in both types of tissue (Fig. 4A, B)  
341 were immunopositive to an antibody against P2Y<sub>12</sub> receptors (Moore et al., 2015; Mildner et al.,  
342 2017). P2Y<sub>12</sub> staining was detected, typically throughout microglia membrane and cytoplasm, for all  
343 amoeboid cells ( $n=25$ ) and all ramified microglia ( $n=33$ ) from MTLE-HS or PTC tissue (Fig. 4C).

344 In contrast, fewer Iba1<sup>+</sup> microglia were immunopositive for A<sub>2A</sub> receptors and cellular staining was  
345 less uniform (Fig. 4A, B). A<sub>2A</sub> immunostaining was also evident outside microglia, suggesting the  
346 receptor was expressed by other cell types, unlike P2Y<sub>12</sub> staining. A<sub>2A</sub> and Iba1<sup>+</sup> immuno-staining  
347 were both detected in 13 of 25 amoeboid cells and 7 of 33 ramified microglia from a MTLE-HS or PTC  
348 tissue (Fig. 4C).

349 We also asked whether membrane distributions of these receptors altered during process extension  
350 or retraction induced by purinergic agonists. For microglia fixed in the resting state, P2Y<sub>12</sub> receptor  
351 immunostaining was most intense at junctions between processes or towards process extremities  
352 (Fig. 4D). When slices were fixed after process extension induced by low doses of ADP, the highest  
353 intensities of immunostaining were detected at the bulbous tips of microglial processes (Fig. 4E).

354 (Figure 4: immuno: A<sub>2A</sub>R, P2Y<sub>12</sub>R. amoeboid cell, ramified microglial)

355

356

357

358 **Purine receptor sub-type underlying process extension.**

359 We sought pharmacological evidence that the activation of distinct purinergic receptors underlies  
360 extension or retraction of microglial processes. Receptor identification is not simple, since intrinsic  
361 tissue ecto-enzymes can hydrolyze ATP to ADP and ADP to adenosine (Yegutkin, 2014; Dale, 1998).  
362 We therefore used a non-hydrolyzable ADP analog, 2MeSADP (1 nM), which activates P2Y1, P2Y12  
363 and P2Y13 receptors (Abbracchio et al., 2019). Fig. 5A shows 2MeSADP induced process extension of  
364 amoeboid microglia. 2MeSADP application significantly increased cross-sectional area by  $49 \pm 9$  %  
365 ( $p=0.01$ ;  $t=-5.33$   $n=2$  MTLE cells, 2 PTC cells), but did not provoke membrane ruffling ( $n=2$  MTLE cells,  
366 2 PTC cells).

367 Fig. 5B-D shows the effects of the specific P2Y12 antagonist PSB0739 (Baqi et al., 2009). At 0.1-1  $\mu$ M,  
368 PSB0739 application led to a rapid cessation of surveilling movements (Fig. 5C; **Movie 4**) and the  
369 retraction of processes of ramified microglia (Fig. 5D; **Movie 4**). Cross sectional area was significantly  
370 reduced by  $25 \pm 3$  % after PSB0739 application,  $p=0.002$ ;  $t=7.47$ ;  $n=3$  PTC cells,  $n=2$  MTLE cells; Fig.  
371 5B). This data suggests tonically activated P2Y12 receptors contribute to the control of process length  
372 and surveillance movements (Matyash et al., 2017). To confirm this point, we tested Ticagrelor,  
373 another P2Y12 receptor antagonist. Ticagrelor (1-5  $\mu$ M; not shown) also suppressed surveillance  
374 movements of ramified cells. Cross-sectional area was significantly reduced by  $27 \pm 5$ % by Ticagrelor  
375 ( $p=0.01$ ;  $t=5.00$ ;  $n=4$  ramified microglia, all MTLE). Thus P2Y12 receptor antagonists suppress  
376 surveillance movements and reduce microglial process cross-sectional area to an extent comparable  
377 to 2mM ADP.

378 (Movie 4: PSB 0379 suppresses surveillance motility and blocks ADP-induced process extension)

379 We next asked whether PSB0739 (1  $\mu$ M) or Ticagrelor (5  $\mu$ M) affected process extension induced by  
380 10  $\mu$ M ADP (Fig. 5B; Movie 4). In 5 amoeboid microglia, increases in cross-sectional area were  
381 suppressed in the presence of PSB0739 (not shown). Similarly 10  $\mu$ M ADP no longer induced  
382 extension of processes of ramified cells in the presence of PSB0739. The change in cross-sectional  
383 area for 7 cells was  $5 \pm 3$  % ( $p=0.13$ ;  $t=-1.72$ ; Fig. 5B;  $n=7$ ; 5PTC, 2 MTLE; Fig. 7B). Process extension  
384 was also suppressed by Ticagrelor (5 $\mu$ M;  $n=3$  cells; not shown) and extension observed on  
385 withdrawal of 2 mM ADP was also suppressed by PSB0739 ( $n=5$  cells; not shown).

386 In summary, human microglia of amoeboid or ramified form, were all P2Y12+ (Fig. 5). Antagonists of  
387 these receptors blocked process extension and also suppressed surveillance movements revealing a  
388 tonic action of P2Y12 receptor activation.

389 (Figure 5: pharmacology of process extension)

390

391 **Purine receptor sub-type initiating microglial ruffling and process retraction.**

392 We asked whether adenosine receptor activation was involved in microglial process retraction or  
393 membrane ruffling induced by higher doses of purines. The stable broad-spectrum analog, 2-chloro-  
394 adenosine (100  $\mu$ M) was used to activate adenosine receptors. It induced a small reduction ( $8\pm 10\%$ )  
395 in cross-sectional area of ramified microglia ( $p=0.46$ ;  $t=0.79$ ; 2 PTC, 4 MTLE cells; Fig. 6A), but did not  
396 induce membrane ruffling of amoeboid microglia (2 PTC, 4 MTLE cells).

397 Next we tested the effects of specific A2A receptor antagonists Preladenant and SCH58261 on the  
398 process retraction induced by 2 mM ADP. In 5  $\mu$ M Preladenant, ADP application significantly reduced  
399 microglial cross-sectional area by  $36\pm 3\%$  ( $p=0.001$ ;  $t=10.33$   $n=4$  PTC cells Fig. 6B). Similarly SCH58261  
400 (5  $\mu$ M) did not prevent process retraction induced by ADP. 2 mM ADP reduced cross sectional area  
401 by  $47\pm 9\%$  in the presence of SCH58261 ( $p=0.03$ ;  $t=5.13$ ; 3 PTC microglia). In summary, not all  
402 microglia, including amoeboid cells, express A2A receptors (Fig. 5). Antagonists of these receptors did  
403 not suppress process retraction induced by strong purinergic stimuli.

404 If adenosine receptors are not involved in process retraction, might P2Y receptors be responsible?  
405 We turned to another broad spectrum agonist, the non-hydrolyzable ATP analog, 2MeSATP which  
406 activates P2Y1, P2Y13 and P2Y6 but not P2Y12 receptors (Abbracchio et al., 2019). 2MeSATP (100  
407  $\mu$ M) reduced microglial cross-sectional area significantly by  $31\pm 6\%$  ( $p=0.04$ ;  $t=4.73$ ; 3 ramified PTC  
408 microglia; Fig. 6C). Membrane ruffling was initiated in all 3 amoeboid PTC microglia tested.

409 These data suggest that 2MeSATP activates a distinct purinergic receptor which is responsible for the  
410 effects of strong ADP stimuli. In experiments with specific P2Y receptor antagonists, we found the  
411 P2Y6 antagonist MRS 2578 (10  $\mu$ M) did not block the ADP 2mM induced process retraction (4  
412 ramified PTC cells, not shown). The P2Y1 antagonists MRS2179 (5-10  $\mu$ M) and MRS2500 (10  $\mu$ M) did  
413 not suppress process withdrawal. A reduction of  $33\pm 5\%$  in mean cross-sectional area was induced by  
414 2 mM ADP applied in the presence of the antagonists ( $p=0.002$ ;  $t=6.50$ ;  $n=5$ , 3 MTLE and 2 PTC cells;  
415 Fig. 6D). The P2Y13 antagonist MRS2211 (10  $\mu$ M) did not suppress ADP mediated retraction of  
416 processes of ramified cells. Mean cross-sectional area was reduced by  $31\pm 6\%$  by 2 mM ADP  
417 application in the presence of MRS2211 ( $p=0.01$ ;  $t=4.97$ ;  $n=4$  PTC cells; Fig. 6E). Furthermore  
418 membrane ruffling induced in amoeboid microglia by 2 mM ADP persisted in the presence of  
419 MRS2211 ( $n=4$  PTC cells).

420 However, joint application of P2Y1 and P2Y13 antagonists suppressed process retraction (MRS2500  
421 and MRS2211; Fig. 6F, G; **Movie 5**). In the presence of both antagonists, mean cross section area  
422 changed by only  $5\pm 7\%$  in response to 2 mM ADP ( $p=0.18$ ;  $t=1.62$ ;  $n=5$  ramified cells; 2 MTLE, 3 PTC  
423 microglia Fig. 6G). In 3 of 5 initially ramified cells, some microglial processes retracted and others

424 extended but overall cross sectional area was little changed. Microglial perisomatic volume tended  
425 to increase (Fig. 6G) and membrane ruffling was suppressed in the presence of both antagonists.

426 (Figure 6: pharmacological basis of high-dose process retraction)

427 (Movie 5: Co-application of P2Y1 and P2Y13 receptor antagonists suppresses process retraction)

428 We next sought anatomical support for a possible role of P2Y1 and P2Y13 receptors in microglial  
429 process retraction using immunohistochemistry to test their expression in microglia. Microglia were  
430 identified by Iba1 immunostaining together with P2Y1 and P2Y13 receptor antibodies (Methods).  
431 Expression was examined for 95 Iba1+ cells (47 ramified, 48 amoeboid) in 6 PTC slices from 3 patients  
432 (Fig 6-1). P2Y1+ immunostained 70 % of Iba1+ cells (76% ramified, 62% amoeboid cells). P2Y13  
433 immunopositivity was expressed by 63 % of Iba1+ cells (80% ramified, 45% amoeboid cells). Both  
434 P2Y1 and P2Y13 immunostaining were detected outside Iba1+ cells, suggesting other cell types may  
435 express these receptors.

436

437 (Fig 6-1: P2Y1, P2Y13 immunohistochemistry on microglia)

438

#### 439 **Microglial process extension and retraction in response to neuronal ablation.**

440 Finally we used laser ablation to examine the effects of tissue damage on the process motility of local  
441 microglia (Davalos et al., 2005; Eyo et al., 2014). Since damage induces purine release (possibly with  
442 other mediators), this approach let us test the legitimacy of pharmacological data. Bright field images  
443 from targeted regions with initially visible neurons were examined before and after stimulation to  
444 monitor effects of laser ablation.

445 In response to laser damage all microglia (4 amoeboid and 3 ramified PTC and MTLE cells) extended  
446 1-3 novel processes. They projected 15-30  $\mu\text{m}$  towards the damage site and encircled the damaged  
447 region (Fig. 7A; **Movie 6**). As in responses to ADP, the tips of extending processes terminated in  
448 bulbous endings. Phagocytic cup-like structures were sometimes apparent. Process extension was  
449 initiated at  $\sim 3$  min after laser stimulation and ceased at  $\sim 10$  min. Mean microglial cross sectional area  
450 increased by  $52 \pm 14$  % (  $p = 0.01$ ;  $t = -3.62$ ; 7 PTC and MTLE cells). This convergence of extending  
451 microglial processes resembles responses to neuronal damage (Petersen & Dailey, 2004), NMDA  
452 receptor activation (Dissing-Olesen et al., 2014) or low calcium levels (Eyo et al., 2015).

453 We next examined the effects of the P2Y12 antagonist PSB 0739 (1  $\mu\text{M}$ ) on process extension  
454 induced by laser damage (4 initially amoeboid cells PTC, 3 ramified cells, PTC). PSB 0739 induced  
455 process retraction and membrane ruffling (as Fig. 5B). A further laser stimulation at the same site, in

456 the presence of the antagonist, no longer triggered process extension, but rather retraction and  
457 membrane ruffling (4 amoeboid and 3 ramified cells, PTC; Fig. 7B; **Movie 6**).

458 If the P2Y12 antagonist suppresses process extension towards a damaged site, extension should be  
459 restored in its absence. We tested this point with a second stimulation at the same site with  
460 observations on 4 PTC and 2 MTLE cells. In response to the second laser stimulus, mean process cross  
461 sectional area increased by  $47 \pm 18\%$  ( $p = 0.04$ ;  $t = -2.57$ ; 4 PTC cells, 2 MTLE cell; **Movie 7**). The movie  
462 shows processes with bulbous endings at their tips approach and encircle the damaged site.

463 (Figure 7: microglial process responses to laser ablation)

464 (Movie 6: Local laser damage induces process extension, blocked by P2Y12R antagonist)

465 (Movie 7: Process extension restored in the absence of P2Y12R antagonist)

466

467

## 468 DISCUSSION

469

470 This work examined purinergic effects on processes of microglia from patients with MTLE and with  
471 glioma, both associated with an inflammatory state. Purines induced process extension at low doses  
472 and process retraction at higher doses. Process extension, for both ramified and amoeboid microglia,  
473 resulted from the activation of P2Y12 receptors. Laser induced tissue damage reproduced microglial  
474 process extension. P2Y12 receptors also tonically promoted microglia process surveillance  
475 movements. Our data suggest joint activation of P2Y1 and P2Y13 receptors underlies process  
476 retraction and ruffling behaviors initiated by higher intensity purinergic stimuli.

477

### 478 ***Microglia in a pathological context: response to low and high intensity purinergic stimuli***

479 Immunostaining showed most, possibly all, MTLE and PTC microglia expressed the P2Y12 receptor,  
480 which triggers process extension in rodents (Haynes et al., 2006; Ohsawa et al., 2010; Dissing-Olesen  
481 et al., 2014). A minority of microglia were immuno-positive for the A2A receptor which we supposed  
482 might initiate process retraction (Orr et al., 2009; Gyoneva et al., 2014). Motility responses were  
483 consistent with these data. Process extension by both ramified and amoeboid microglia initiated by  
484 weak purinergic stimuli was suppressed by antagonists of P2Y12 receptors. Process retraction  
485 induced by stronger stimuli was not suppressed by A2A receptor antagonists, but was blocked by  
486 joint application of P2Y1 and P2Y13 receptor antagonists.

487 In rodents, surveilling, ramified cells and amoeboid, 'activated' microglia respond differently to  
488 purinergic stimuli. Microglia 'activated' by lipopolysaccharide (LPS) treatment, retract rather than  
489 extend their processes. Microglial expression of P2Y12 receptors is down-regulated after LPS  
490 treatment (Haynes et al 2006; Orr et al., 2009). A2A receptors are up-regulated and trigger retraction  
491 (Gyoneva et al 2014; Orr et al., 2009). Our data shows microglia in tissue from patients with MTLE or  
492 gliomas do not behave in this way.

493 How similar are the phenotypes of 'activated' human epileptic microglia and LPS-treated rodent  
494 cells? Microglia of epileptic human tissue were first classed as 'reactive' according to shape and  
495 inflammatory marker expression (Beach et al., 1995). We now know seizures release inflammatory  
496 molecules (Vezzani et al., 1999; Morin-Brureau et al., 2018) and rupture the blood-brain barrier  
497 (Cornford, 1999; Cacheaux et al., 2009) permitting entry of systemic immune cells. Our data showed  
498 microglia of the CA1 region in MTLE tissue or closer to the tumor in PTC tissue were more likely to  
499 possess an 'activated' shape with few or no processes. However P2Y12 receptor expression by cells  
500 of all shapes (Fig. 4) seems inconsistent with other human data. Microglia close to lesions in tissue of

501 multiple sclerosis patients (Mildner et al 2017; Zravy et al., 2017) or near amyloid tangles in patients  
502 with Alzheimers disease (Mildner et al., 2017) show little or no P2Y12 immunostaining. We note the  
503 antibody used here recognizes the same 40-amino acid cytoplasmic region of the receptor as that  
504 used by Mildner et al. (2017). Data showing all Iba1+ elements were P2Y12+ (cf. Mildner et al. 2017)  
505 suggests that cells studied here were microglia rather than infiltrating immune cells.

506 However microglia 'activated' in different contexts may exhibit distinct motility patterns and express  
507 different receptors. 'Alternatively activated' human microglia, treated with interleukins IL-4 and IL13  
508 rather than LPS, show more intense immunostaining against P2Y12 and respond more strongly to  
509 ADP (Moore et al., 2015). 'Alternatively activated' microglia are linked to repair processes, as  
510 transcriptomic data from the CA1 region of MTLE tissue suggests (Morin-Brureau et al, 2018). In  
511 kainite-treated epileptic mice, 'activated' microglia continue to extend processes towards purinergic  
512 stimuli or cellular damage (Avignone et al., 2015). The phenotype of 'activated' microglia in human  
513 epileptic brain may not be identical to that of LPS-treated rodent cells.

514

515 ***P2Y12R: Microglial process extension and basal effects on surveillance.***

516 We found few differences between ramified and amoeboid microglia in the process extension  
517 induced by low intensity purinergic stimuli (Haynes et al., 2006; Ohsawa et al., 2010; Dissing-Olesen  
518 et al., 2014). Increases in cross-sectional area were large, 80-100 % for initially amoeboid cells,  
519 possibly mediated by endocytic membrane recycling (Kay et al., 2008). Processes were directed  
520 towards purinergic stimuli and P2Y12 receptors moved toward growth sites (Dissing-Olesen et al.,  
521 2014). Microglia of MTLE and PTC tissue exhibited similar motility responses. Similar, even though  
522 ambient glutamate levels in PTC are higher than in MTLE tissue (Buckingham et al., 2011) and mitosis  
523 and cell cycle-related molecules are upregulated in peritumoral microglia (Szulzewsky et al. 2016).  
524 Specific tumor associated macrophages may have immunosuppressive effects in glioblastoma (Chen  
525 & Hambardzumyan, 2018) and immune related transcripts of human microglia (Szulzewsky et al.,  
526 2016) are not notably upregulated in glioblastoma tissue.

527 Actions of the antagonists PSB0739 and Ticagrelor, suggests tonic activation of the P2Y12 receptor  
528 promotes non-directed surveillance movements. Similar conclusions have emerged from work on  
529 spinal microglia of mice genetically deficient for the P2Y12 receptor (Gu et al., 2016), but not from  
530 rodent hippocampal microglia treated with receptor antagonists (Madry et al., 2018). A role for  
531 P2Y12 receptors in directed extension of rodent microglial processes is generally agreed (Haynes et  
532 al., 2006; Madry et al., 2018). although adenosine receptors may also need to be activated (Matyash  
533 et al., 2017). Pathways linking P2Y12 receptor activation to process extension remain to be

534 completely described. This receptor is coupled to  $G_{\alpha i}$  (Erb & Weisman, 2012; Abbracchio et al., 2019).  
535 Activation inhibits adenylate cyclase, increases internal Ca and induces K-currents. Those mediated  
536 by the 2-pore channel, THIK1, contribute to surveillance movements but not process extension  
537 (Madry et al., 2018). Akt, a Ca-dependent kinase, activates beta1 integrins facilitating process  
538 extension by interactions with the extracellular matrix (Somanath et al 2007; Ohsawa et al, 2010).  
539 The PI3k pathway has been implicated in purine-induced chemotaxis (Wu, Vadakkan & Zhuo, 2007).  
540 Small GTPase pathways are crucial modulators of cytoskeletal actin remodeling (Sadok & Marshall,  
541 2014; Rottner et al., 2017) but their involvement in microglial process extension is not clear.

542

### 543 ***P2Y1/P2Y13: joint effects on microglial process retraction and ruffling***

544 Our data suggest that P2Y1 and P2Y13 receptors are involved in microglial process retraction induced  
545 by high dose purinergic stimuli, the same as used previously in rodents (Madry et al., 2018; Eyo et al.,  
546 2014). Ramified cells of both MTLE and PTC behaved similarly. In contrast to extension which was  
547 directed, retraction was non-directional. Membrane ruffling of ramified and amoeboid cells, induced  
548 by high intensity stimuli was also suppressed by simultaneously applied P2Y1 and P2Y13 antagonists.

549 Neither P2Y1 nor P2Y13 receptors have been linked to microglial process retraction. The P2Y1  
550 receptor is coupled to  $G_{\alpha q}$  and activates phospholipase C as well as the small GTPases Rac and Rho  
551 (Erb & Weisman, 2012; Abbracchio et al., 2019). The P2Y13 receptor is strongly homologous to  
552 P2Y12, coupled to the same  $G_{\alpha i}$  and also inhibits adenylate cyclase as well as RhoA, a small GTPase.  
553 Links between P2Y1 and P2Y13 receptors, actin filament remodeling and process retraction remain to  
554 be explored.

555 The finding that both P2Y1 and P2Y13 antagonists were needed to suppress process retraction might  
556 imply a synergy between signaling pathways initiated by distinct receptors or rather reflect the  
557 activation of a single receptor entity. Precedents exist for either possibility. For instance, there is  
558 reciprocal cross-talk between intracellular pathways activated by P2Y1 and P2Y12 receptors in  
559 platelets (Hardy et al., 2004). Alternatively, distinct G-protein coupled receptors may express as  
560 dimers (Gurevich & Gurevich, 2008). P2Y1 receptors form heteromers with both P2Y11 (Ecke et al.,  
561 2008) and A1R adenosine receptors (Yoshioka, Saitoh & Nakata, 2001). Immunostaining data  
562 (Extended Fig. 4) suggested that P2Y1 and P2Y13 were expressed at higher levels in ramified than  
563 amoeboid microglia but not universally, unlike P2Y12 receptors. Staining could seem to overlap on  
564 Iba1+ cells, raising the possibility that both antibodies recognized receptors involved in retraction.

565

566

567 ***Functional significance***

568 Our data on microglial process movements induced by focal tissue damage reinforces results from  
569 purine applications, although we did not measure levels of purines or adenosine (Laudet et al., 2005).  
570 Epileptiform activity induced in slices causes adenosine increases in the range of 10  $\mu$ M with smaller  
571 changes in ATP (Frenguelli & Wall, 2016). Extracellular purine levels may be much higher after brain  
572 damage in part due to ATP release from astrocytes (Franke, Krügel & Illes P, 2006; Choo et al., 2013).

573 Microglia adopt shapes ranging from 'reactive' ovoid cells through 'activated' microglia with large  
574 soma and some thick processes to highly ramified cells (Fig. 8). We suggest that purinergic signaling  
575 could partially, but not completely, underlie cycling of a given cell through these different shapes and  
576 back again (Hanisch & Kettenmann, 2007; Yamada & Jinno, 2013; Jonas et al., 2012). Specifically,  
577 weak ADP stimuli, and cell damage, induce strong process extension by amoeboid or even round  
578 microglia. However process retraction of highly ramified cells exposed to high purine levels does not  
579 produce completely amoeboid cells, but rather microglia with shorter ramifications.

580 In conclusion, we have implemented live imaging of microglia in tissue from patients with temporal  
581 lobe epilepsy and cortical glioma. Cell shape varied from highly ramified microglia to cells with few or  
582 no processes. Low levels of purinergic stimuli intensity induced process extension of all microglia  
583 mediated via P2Y<sub>12</sub> receptors. Higher purine doses induced retraction of ramified microglial  
584 processes and membrane ruffling which were suppressed by joint application of P2Y<sub>1</sub> and P2Y<sub>13</sub>  
585 receptor antagonists.

586

587

588 **FIGURE LEGENDS**

589

590 **Figure 1. Staining and form of microglia in human peritumoral cortex and temporal lobe.**

591 **A.** Staining of peritumoral cortex with IB4 lectin (green, left), tomato lectin (red, middle) and both  
592 signals (merge). Maximal intensity from z-stack (total 50  $\mu\text{m}$ , step 1  $\mu\text{m}$ ) at 10 min after focal  
593 injection of lectins. IB4 lectin stained only endothelial cells of blood vessels (BV). Tomato lectin  
594 stained both microglia and endothelial cells. **B.** Tomato lectin positive cells (right) were also Iba1+  
595 (left). **C.** Tomato lectin labeled microglia from peri-tumoral cortex and from the CA1, dentate and  
596 subicular regions of MTLE tissue. Maximal intensity from z-stacks (50  $\mu\text{m}$ , 1  $\mu\text{m}$  step). **D.** 3-D  
597 reconstructions of cells in C. Cross-sectional area at upper right. **E.** Cross sectional area distributions  
598 for cells of peri-tumoral cortex (n=34) and CA1 (n=25), dentate gyrus (n=17) and subiculum (n=18  
599 cells) of MTLE tissue. The red line is the threshold of 300  $\mu\text{m}^2$  used to distinguish amoeboid and  
600 ramified cells.

601

602 **Figure 2: At low doses ADP induces microglial process extension**

603 **A.** Tomato lectin stained PTC microglia before (CTRL, left) and after bath application of ADP (10  $\mu\text{M}$ ,  
604 15 min, right). Change maximal cross sectional area are shown in red. (z-stacks 50  $\mu\text{m}$ , 1  $\mu\text{m}$ ). **B.**  
605 Lateral view shows microglial processes extended toward the upper surface of the slice. Red arrows  
606 show bulbous endings at their tips. **C.** ADP at low doses induces process emergence and extension  
607 from an amoeboid cell. Images are control (CTRL, left) and ADP (10  $\mu\text{M}$ , 15 m, middle). Merge (right)  
608 shows color-coded differences between CTRL and ADP images. Red, new signal; green, lost signal;  
609 yellow, maintained signal (z-stack 20  $\mu\text{m}$ , 1  $\mu\text{m}$ ). Inset at right shows bulbous endings (red arrow) at  
610 the tips of processes. **D.** ADP induces process extension of a ramified microglia. Control (CTRL, left),  
611 ADP (10  $\mu\text{M}$ , 15 min middle) and color-coded differences between them (Merge, right). Red, new  
612 signal; green, lost signal; yellow, maintained signal. (z-stack 20  $\mu\text{m}$ , 1  $\mu\text{m}$ ). The inset shows bulbous  
613 endings (red arrow) at process tips. **E.** Time course of changes in mean cross-sectional area induced  
614 by 10  $\mu\text{M}$  ADP in 7 amoeboid microglia. **F.** Time-course of changes in mean cross-sectional area  
615 induced by 10  $\mu\text{M}$  ADP in 7 ramified microglia. **G.** Mean and s.e. of the latency to 20 % increase in  
616 cross sectional area induced by ADP for amoeboid (n=7) and ramified microglia (n=7). Process  
617 extension by ramified cells occurred at a significantly longer latency (t-test, p=0.009).  
618 Figure 2-1 shows the changes in cross-sectional area for ADP doses of 2  $\mu\text{M}$ –2 mM. Figure 2-2 shows  
619 the ADP effects on ramified and amoeboid microglia of PTC and MTLE tissue. **Movie 1 shows process**  
620 **extension of an amoeboid cell induced by low-dose ADP.**

621

622

623 **Figure 2-1: Dose- response for process motilities induced by ADP**

624 **A.** Percentage changes in microglial cross-sectional area induced by ADP (1  $\mu$ M – 2 mM). Ramified  
625 cells, filled diamonds; amoeboid microglia, open squares. Extension was maximal for 10  $\mu$ M ADP. **B.**  
626 Changes of cross-sectional area on ADP washout for initially ramified (filled diamonds) and initially  
627 amoeboid cells (open squares) plotted as percentage change from area under ADP. Minor process  
628 extensions only were detected after exposure to ADP levels less than 2 mM. Absolute changes in  
629 microglial cross-sectional area induced by, **C**, 1-10  $\mu$ M, **D**, 100-500  $\mu$ M, and, **E**, 2 mM ADP. Dashed  
630 line at 300  $\mu$ m<sup>2</sup> is the threshold separating amoeboid and ramified microglia. Amoeboid and ramified  
631 cells behaved in a similar way.

632

633 **Figure 2-2: ADP effects on ramified and amoeboid microglia of PTC and MTLE tissue**

634 **A.** 10  $\mu$ M ADP induces similar process extensions for ramified microglia of PTC (n=4) and MTLE tissue  
635 (n=3). **B.** Low dose ADP (10  $\mu$ M) induces comparable process extensions for amoeboid cells of PTC  
636 (n=4) and MTLE tissue (n=3). **C.** 2 mM ADP induces similar process retractions for ramified microglia  
637 of PTC (n=4) and MTLE (n=5) tissue. Mean responses of MTLE cells shown as open squares and PTC  
638 cells as filled squares.

639

640

641 **Figure 3: High doses of ADP induce microglial process retraction and membrane ruffling.**

642 **A.** ADP induces membrane ruffling of an amoeboid microglia. Control (left), ADP (2 mM, 20 min,  
643 middle). Merge (right) shows color-coded differences. Red, new fluorescent signal; green, lost signal;  
644 yellow, maintained signal. **B.** Transition from 2 mM ADP back to control for the same cell (wash,  
645 left), induces process extension. Merge (right) shows differences from ADP. Blue, new signal; red, lost  
646 signal. **C.** ADP induces process retraction and membrane ruffling of a ramified microglia. Control  
647 (left), ADP (2 mM, 20 min, middle). Merge (right) shows differences. Blue, new signal; green, lost  
648 signal; yellow, maintained signal. **D.** Transition from ADP back to control for the cell in **C** induces  
649 process extension (wash, left). Merge image (right) shows blue, new signal; red, lost signal. **A-D** are  
650 all maximal projections from z-stacks (50  $\mu$ m, 2  $\mu$ m step). **E.** Time course of changes in mean cross-  
651 sectional area in 7 initially amoeboid cells. Little change occurs during application of 2 mM ADP  
652 application. Processes extend when ADP is removed. **F.** Time course of the reduction in mean cross-

653 sectional area induced by 2 mM in 9 ramified microglia. **G.** Different latencies to process extension  
654 (increase of 20 % for cross sectional area).during the transition from 2 mM ADP back to control ACSF.  
655 The latency is significantly longer for ramified microglia (t-test, p=0.004). Figure 3-1 shows the  
656 clearance of a fluorescent ADP from the slice. **Movie 2 shows membrane ruffling of an amoeboid**  
657 **microglia induced by 2 mM ADP.** **Movie 3 shows process retraction of a ramified microglia induced by**  
658 **2 mM ADP.**

659

### 660 **Figure 3-1: Fluorescent ADP clearance from the slice**

661 **A.** Fluorescence at 0, 5, 10, and 20 min after focal ejection of the fluorescent purine EDA-ADP - ATTO-  
662 488 at 1 mM from a patch pipette into middle regions of a 300  $\mu$ m thick PTC slice. **B.** Typical time  
663 course for the decay of fluorescence, measured as whole screen fluorescent signal. **C.** The decay  
664 followed a double exponential time course with fastest decay in the first 3-5 min. The fit was  $y =$   
665  $435.0 \cdot \exp(-x/0.9) + 158.9 \cdot \exp(-x/3.4) + 14.6$ , where  $y$  is fluorescence and  $x$  is time . Fluorescence  
666 was maintained longer than 20 min after ejection into the slice, but decayed in 2-3 sec after ejection  
667 into the bath.

668

### 669 **Figure 4: Expression of P2Y12 and A2A receptors by human microglia.**

670 **A.** Immunostaining against P2Y12 and A2A receptors in an IBA1+, ramified PTC microglia. **B.** P2Y12  
671 and A2A receptor immunostaining for an amoeboid IBA1+ cell from MTLE tissue. Panels in **A** and **B**  
672 are from the left: P2Y12 immuno-staining (green), IBA1 (white), A2A (red) and merged P2Y12 and  
673 A2A staining. **C.** Proportion of ramified (clear; n=33) and amoeboid (hatched; n=25) microglia  
674 immuno-positive for P2Y12 (gray) and A2A receptors (red). **D.** Color-coded staining intensity (green,  
675 low – red, high) shows P2Y12 staining was highest towards the tips of processes of a ramified  
676 microglia (above) in the resting state. Intensity was highest in a somatic region of a non-stimulated  
677 amoeboid cell (below). **E.** Color-coded intensity (green low – red high) for P2Y12 immunopositivity  
678 for an initially amoeboid microglia fixed at full extension induced by 10  $\mu$ M ADP. Staining intensity  
679 was low in peri-somatic regions (above) and highest at bulbous endings of processes (below).

680

### 681 **Figure 5: P2Y12 receptors and microglial motilities.**

682 **A.** The non-hydrolyzable ADP analog, 2MeSADP (1 nM) increased mean microglial cross sectional  
683 area (n=4 PTC cells). **B.** The P2Y12 receptor antagonist PSB0739 (1  $\mu$ M) reduced the mean cross-  
684 sectional area of ramified microglia (3 PTC and 2 MTLE microglia). It prevented the process extension  
685 induced by 10  $\mu$ M ADP. **C.** PSB0739 suppressed surveillance motility of processes of a ramified

686 microglia. Right image control. Left image, process positions at 5 min intervals during control period  
687 shown by colors (green, red, yellow, dark blue, light blue). **D.** Right, after PSB0739, Middle, processes  
688 in the presence of PSB 0739 (1  $\mu$ M) and ADP (10  $\mu$ M). Left, process positions at 5 min intervals (colors  
689 as in C). **Movie 4 shows that PSB 0379 suppresses surveillance motility and blocks ADP-induced**  
690 **process extension.**

691

#### 692 **Figure 6: Receptors involved in microglial process retraction.**

693 **A.** The stable adenosine analog 2-chloro-adenosine (100  $\mu$ M) induced a small reduction in mean  
694 microglial cross-section area (4 MTLE and 2 PTC ramified cells). **B.** The A2A receptor antagonist,  
695 Preladenant (5  $\mu$ M) did not suppress process retraction induced by 2 mM ADP (n=4 ramified PTC  
696 cells). **C.** The non-hydrolyzable ATP analog, 2MeSATP (100  $\mu$ M) reduced the mean cross sectional  
697 area of 3 ramified microglia of MTLE tissue. **D.** P2Y1 receptor antagonists MRS2500 or MRS2179 (10  
698  $\mu$ M) did not prevent the reduction of microglial cross-sectional area induced by 2 mM ADP in  
699 ramified microglia (4 MTLE, 2 PTC cells). **E.** The P2Y13 antagonist MRS2211 (30  $\mu$ M) did not suppress  
700 process retraction induced by 2 mM ADP in 4 ramified PTC microglia. **F.** Co-application of MRS2500  
701 (10  $\mu$ M) and MRS2211 (30  $\mu$ M) suppressed retraction induced by 2 mM ADP 2mM (5 ramified cells; 2  
702 MTLE, 3 PTC cells). **G.** Response of an initially ramified microglia (PTC) to 2 mM ADP in the presence  
703 of MRS2500 (10  $\mu$ M) and MRS2211 (30  $\mu$ M). From the left CTRL, basal state, in the presence of both  
704 antagonists and then 2 mM ADP. The merge image shows the difference between cell shape in the  
705 presence of both antagonists and then after applying ADP. New fluorescent signal, green; lost signal  
706 red; maintained signal, yellow. Figure 6-1 shows the immunohistochemistry for P2Y1 and P2Y13  
707 receptors on fixed slices. **Movie 5 shows that the co-application of P2Y1 and P2Y13 receptor**  
708 **antagonists suppresses process retraction.**

709

#### 710 **Figure 6-1: P2Y1 and P2Y13 immunostaining**

711 **A.** Immunostaining against P2Y1 and P2Y13 receptors in an IBA1 + microglia (amoeboid cell of PTC).  
712 **B.** P2Y1 and P2Y13 receptor immunostaining for a ramified PTC cell. Panels in **A** and **B** are from the  
713 left: Iba1 staining (green), P2Y1 staining (blue), P2Y13 staining (red) and merge of P2Y1, P2Y13 and  
714 Iba1 staining. Arrows indicate the spatial overlap of P2Y1 and P2Y13 staining in Iba1+ cells in both **A**  
715 and **B**. Such overlap was seen in 47 % (45/95) of Iba1+ microglia **C.** Ramified cells (n=47) were more  
716 likely than amoeboid cells (n= 48) to be immunopositive for P2Y1 (blue) or P2Y13 receptors (red).

717

718 **Figure 7: Role of P2Y receptors in microglial motility induced by tissue damage.**

719 **A.** The effects of local laser stimulation. Control images of an initially amoeboid cell from peri-  
720 tumoral cortex before (CTRL), immediately after laser damage (yellow arrow) and at 10 and 15 min  
721 later. Merge is the difference between control and +15 min images. New membrane signal in red,  
722 lost signal in green. **B.** The same cell after a second laser stimulus (yellow arrow) applied 5 min after  
723 the P2Y12 antagonist PSB 0739 (1  $\mu$ M). Images are shown at 5 and 15 min after stimulation. Merge is  
724 the difference between PSB0739 and 15 min images. Lost membrane signal due to process retraction  
725 in red and new membrane signal from ruffling in blue. **Movie 6 shows that local laser damage**  
726 **induces process extension and is blocked by P2Y12R antagonist. Movie 7 show that process**  
727 **extension is restored in the absence of P2Y12R antagonist.**

728

729

730 **Figure 8: Schema of microglial shape changes and P2Y receptors.**

731 **A.** Scheme showing the effect of purines of microglial morphologies. Amoeboid and ramified cells are  
732 basal microglial states. Microglia change shape between states accordingly to levels of ATP/ADP.

733

734

735

736 **MOVIE LEGENDS**

737

738 **Movie 1:** Process extension of an initially amoeboid, tomato lectin labeled microglia from peri-  
739 tumoral cortex induced by 10  $\mu$ M ADP applied at time counter = 10 min. Scale, bottom left. Frame  
740 rate 3 per sec.

741

742 **Movie 2:** Membrane ruffling of an initially amoeboid cell from the sclerotic CA1 region of MTLE tissue  
743 on application of 2 mM ADP, at time counter = 5.40 min. Followed by process extension when ADP  
744 was removed, at time counter = 45.25 min. Scale, bottom left. Frame rate 3 per sec.

745

746 **Movie 3:** Process retraction and membrane ruffling of an initially ramified cell from peri-tumoral  
747 cortex induced by application of 2 mM ADP at time counter=7.13 min. Followed by vigorous process

748 extension when ADP was removed, at time counter = 49.04 min. Scale, bottom left. Frame rate 3 per  
749 sec.

750

751 **Movie 4:** Process retraction and cessation of surveillance motility of a ramified microglia of peri-  
752 tumoral cortex induced by PSB 0739 (1  $\mu$ M) applied at time counter= 11 min. ADP at 10  $\mu$ M applied  
753 in the presence of PSB0739 at time counter =25 min does not induce process extension. Scale,  
754 bottom left. Frame rate 3 per sec.

755

756 **Movie 5:** Joint application of P2Y1 (MRS2500, 10  $\mu$ M) and P2Y13 (MRS2211, 30  $\mu$ M) antagonists, at  
757 time counter =11 min, suppresses ADP-induced (2 mM) process retraction of an initially ramified  
758 microglia from the dentate gyrus of an MTLE tissue. ADP applied at time counter = 30.00 min. Scale,  
759 bottom left. Frame rate 3 per sec.

760

761 **Movie 6:** Laser-induced tissue damage induces process extension by an initially amoeboid microglia  
762 of peri-tumoral cortex. Red circle indicates laser damage. First laser stimulus at time counter=2.59  
763 min. PSB 0739 (1  $\mu$ M) was applied at time counter=16.59 min. Second laser stimulus of the same  
764 intensity at the same site, at time counter=21.59 min. Scale, bottom left. Frame rate 3 per sec.

765

766 **Movie 7:** Two laser stimuli at interval 7 minutes, both induce process extension by 2 initially  
767 amoeboid and 1 ramified microglia of peri-tumoral cortex. The green arrow points to the targeted  
768 region and the red circle indicates the visible extent of damage. First laser stimulation at time 3.00  
769 min and the second at time counter 10.37 min. Scale, bottom left. Frame rate 3 per sec.

770

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