The P2X7 receptor localizes to the mitochondria, modulates mitochondrial energy metabolism and enhances physical performance

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18 **Abstract**

19 Basal expression of the P2X7 receptor (P2X7R) improves mitochondrial metabolism, ATP
 20 synthesis and overall fitness of immune and non-immune cells. We investigated P2X7R
 21 contribution to energy metabolism and subcellular localization in fibroblasts (mouse embryo
 22 fibroblasts and HEK293 human fibroblasts), mouse microglia (primary brain microglia and the N13
 23 microglia cell line), and heart tissue. The P2X7R localizes to mitochondria, and its lack a) decreases
 24 basal respiratory rate, ATP-coupled respiration, maximal uncoupled respiration, resting
 25 mitochondrial potential, mitochondrial matrix Ca^{2+} level, b) modifies expression pattern of
 26 oxidative phosphorylation (OxPhos) enzymes, and c) severely affects cardiac performance. Hearts
 27 from *P2rx7*-deleted versus WT mice are larger, heart mitochondria smaller, and stroke volume
 28 (SV), ejection fraction (EF), fractional shortening (FS) and cardiac output (CO), are significantly
 29 decreased. Accordingly, physical fitness of P2X7R-null mice is severely reduced. Thus, the P2X7R
 30 is a key modulator of mitochondrial energy metabolism and a determinant of physical fitness.

31 **Introduction**

32 Adenosine 5'-triphosphate (ATP), the universal energy currency, sustains all cellular functions and
 33 responses, and at the same time is also an extracellular messenger involved in cell-to-cell
 34 communication in virtually every tissue (Burnstock 2014). As an extracellular messenger, ATP is of
 35 remarkable importance at sites of cell damage or distress since it is one of the earliest and most
 36 important damage-associated molecular patterns (DAMPs) released (Bours et al. 2006, Bours et al.
 37 2011). Due to this dual role, as a DAMP and a high energy intermediate, changes in the
 38 extracellular ATP concentration in response to pathogens or to sterile injury might serve not only as
 39 alarm signals but also as triggers to activate cellular energy synthesis in conditions of high energy
 40 demand. This would make cells more reactive to the impending danger.

41 Virtually all cells are equipped with plasma membrane receptors for extracellular ATP, the
 42 P2 receptors (P2Rs) (Burnstock 2006). P2Rs mediate a multiplicity of responses in the nervous,
 43 endocrine, immune and cardiovascular systems. P2Rs are comprised of two subfamilies, the P2Y
 44 metabotropic (P2YR) and the P2X ionotropic (P2XR) receptors, each numbering eight and seven
 45 members, respectively. Within the P2XR subfamily, the P2X7R subtype is of interest for its
 46 peculiar permeability properties and the role in inflammation and cancer (Di Virgilio et al., 2017;
 47 Di Virgilio et al., 2018). This receptor is in fact a very potent activator of the NLRP3
 48 inflammasome, of IL-1 β processing and release (Di Virgilio 2013), as well as a strong promoter of
 49 cancer cell proliferation (Di Virgilio and Adinolfi 2016), and, when over-activated, a trigger of cell
 50 death (Di Virgilio et al. 1989). Survival-promoting effects are likely due to P2X7R capacity to
 51 support oxidative phosphorylation and glycolysis, and thus enhance intracellular ATP synthesis
 52 (Adinolfi et al. 2005, Amoroso et al. 2012). Since during physical activity or at sites of
 53 inflammation the extracellular ATP concentration is several fold increased (Forrester 1966,
 54 Wilhelm et al. 2010), the P2X7R might be considered a bioenergetics sensor enabling the
 55 intracellular ATP synthetic apparatus to sense extracellular ATP levels, and thus meet the increased
 56 energy demand. Along these lines, stimulation of the P2X7R has been previously shown to enhance

57 energy metabolism in mice (Giacovazzo et al. 2019). In this study P2X7R subcellular localization
58 and its effect on mitochondrial energy metabolism was investigated. As cellular models, we used
59 cell types well-known for P2X7R expression and function, i.e. fibroblasts (mouse embryo
60 fibroblasts, MEFs, and P2X7R-transfected HEK293, HEK293-P2X7R, human fibroblasts), and
61 microglia (primary brain microglia and the N13 microglia cell line). Mouse fibroblasts and mouse
62 microglia were isolated from WT or *P2rx7*-deleted mice. N13 microglia was available as the WT
63 cell line or the N13 R cell line selected for low P2X7R expression. Finally, to fully appreciate the
64 functional impact of P2X7R-dependent mitochondrial dysfunction in a tissue heavily dependent on
65 oxidative phosphorylation, we investigated heart performance in WT and P2X7R-deleted mice.
66 Altogether, these data show that the P2X7R localizes to the mitochondria in different cell types, and
67 its lack impairs oxidative phosphorylation, affects cardiac performance and decreases physical
68 fitness.

69 **Results**

70 **Lack of the P2X7R impairs energy metabolism.** In a previous study we showed that P2X7R
 71 expression in human HEK293 fibroblasts increases mitochondrial Ca^{2+} concentration,
 72 mitochondrial membrane potential and overall intracellular ATP content (Adinolfi et al. 2005).
 73 **Figure 1A-C** and **Figure 1E-G** show that P2X7R down-modulation or *P2rx7* gene deletion
 74 decreases resting mitochondrial potential and severely impairs all respiratory indexes in N13
 75 microglia cells, primary mouse microglia, and mouse embryo fibroblasts (MEFs). Accordingly,
 76 overexpression of the P2X7R in cells lacking the endogenous receptor (e.g. human HEK293 cells)
 77 increases mitochondrial potential and improves all respiratory indexes (**Figure 1D,H**). Partially
 78 depolarized mitochondria are anticipated to have lower matrix Ca^{2+} levels as the driving force for
 79 Ca^{2+} is reduced. To verify this prediction we measured mitochondrial Ca^{2+} with the FRET-based
 80 GCaMP indicator. As shown in **Figure 1I**, mitochondrial matrix Ca^{2+} level is significantly lower in
 81 HEK293 WT versus P2X7R-transfected HEK293 cells (HEK293-P2X7R). Previous data showed
 82 that NADH activity is modulated by the mitochondrial matrix Ca^{2+} concentration (Griffiths and
 83 Rutter 2009), thus we verified whether lack of P2X7R is associated with changes in the NADH
 84 concentration. In HEK293-P2X7R cells, higher matrix Ca^{2+} concentration is associated to lower
 85 intra-mitochondrial NADH levels (**Figure J**), and accordingly MEFs from *P2rx7*-deleted mice and
 86 mouse microglia selected for low P2X7R expression (N13R microglia) show higher intra-
 87 mitochondrial NADH levels (**Figure 1K-M**).

Figure 1 Lack of the P2X7R impairs mitochondrial potential, oxygen consumption, calcium level and causes intra-mitochondrial NADH accumulation.

Cells, plated on 12-mm glass coverlips (A,B,C,D), were loaded with TMRM (20 nM) at 37°C for 20 min in KRB buffer. Fluorescence was measured with a Zeiss LS510 confocal microscope. Images were then analyzed with ImageJ software. Mitochondrial membrane potential (Ψ_m) was expressed as the ratio between TMRM fluorescence (in arbitrary units, AU) before and after carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) addition. Exact fluorescence AU values: N13 WT (average $\pm$ SEM = 838.5 $\pm$ 55.96, n = 13) or N13 R (average $\pm$ SEM = 527.9 $\pm$ 66.60, n = 14) (A); MEFs WT (average $\pm$ SEM = 886.6 $\pm$ 59.86; n = 31) or MEFs P2X7-KO (average $\pm$ SEM = 671.5 $\pm$ 35.49, n = 43) (B); primary WT microglia (average $\pm$ SEM = 551.1 $\pm$ 81.53, n = 20) or primary P2X7-KO microglia (average $\pm$ SEM = 134.0 $\pm$ 13.69, n = 8) (C); HEK293-P2X7 (average $\pm$ SEM = 3769 $\pm$ 191.0, n = 47) or HEK293 (average $\pm$ SEM = 2296 $\pm$ 187.7, n = 45) (D). Alternatively, cells were plated in XF96 96-well cell culture plate (E,F,G,H) and analyzed for oxygen consumption in a Seahorse apparatus. Exact averages $\pm$ SEM for panels E, F, G, H are shown in

Supplementary Table I. Oxygen consumption rate (OCR) was normalized on cell content. Data are presented as average $\pm$ SEM of $n = 3$ for panels **E**, **F**, **G** and $n = 7$ for panel **H**. HEK293-P2X7R (average arbitrary fluorescence units $\pm$ SEM = 2717 ± 0.069 , $n = 57$) or HEK293 (average arbitrary fluorescence units $\pm$ SEM = 0.95 ± 0.039 , $n = 58$) cells were transfected with the mitochondrial-selective FRET-based, GCaMP fluorescent Ca^{2+} indicator (**I**), or analysed for NADH autofluorescence with a fluorescence microscope (average arbitrary fluorescence units $\pm$ SEM, HEK293-P2X7R = 23.33 ± 2.07 , $n = 25$; HEK293 = 31.31 ± 2.92 , $n = 31$) (**J**). MEFs WT (average arbitrary fluorescence units $\pm$ SEM = 0.015 ± 0.002 , $n = 11$) or MEFs P2X7R-KO (average arbitrary fluorescence units $\pm$ SEM = 0.026 ± 0.002 ; $p = 0.006$, $n = 23$) (**K**), and N13 WT (average arbitrary fluorescence units $\pm$ SEM = 0.19 ± 0.01 , $n = 24$) or N13 R (average arbitrary fluorescence units $\pm$ SEM = 0.23 ± 0.01 , $n = 37$) (**L**), were also analysed for NADH fluorescence. Fluorescence emission was acquired with an IX-81 Olympus automated epifluorescence microscope as described in Materials and Methods. Intracellular content (pmol/ μL) of NADH for N13 WT (average $\pm$ SEM = 13.18 ± 0.25 , $n = 3$) and N13 R (average $\pm$ SEM = 25.80 ± 0.39 , $n = 3$) (**i**) was also measured with a NAD/NADH Assay Kit, as described in Methods. P values are calculated with the two-tailed unpaired Student's t-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****, $p < 0.0001$.

114 Based on the known Ca^{2+} dependency of mitochondrial dehydrogenases (Griffiths and Rutter 2009),
 115 we anticipated that NADH levels should be lower in the presence of lower matrix Ca^{2+}
 116 concentrations, while in fact they are higher. Thus, we hypothesized that accumulation of NADH in
 117 the absence of P2X7R could be rather due to reduced Complex I activity. To support this
 118 hypothesis, we found that HEK293-P2X7R and P2X7R-sufficient N13 WT cells show increased
 119 Complex I protein levels compared to HEK293-WT and P2X7R-deficient N13 R cells, respectively
 120 (**Figure 2A-D**). It is therefore likely that lower matrix Ca^{2+} in P2X7R-deficient cells is the result of
 121 the reduced mitochondrial potential due to lower Complex I expression, and thus to reduced
 122 respiration as shown in Figure 1. We also observed reduced Complex II expression in HEK293-
 123 P2X7R versus HEK293-WT cells, while no changes in Complex II expression are found in N13
 124 WT versus N13 R cells. We then estimated total mitochondrial content of N13 and MEF cells by
 125 measuring expression of the mitochondrial markers TOM20, TIM23 and Hsp60. As shown in
 126 Supplementary Information 1, mitochondrial markers are not significantly reduced in the absence of
 127 the P2X7R. In N13 cells, reduced expression of the P2X7R decreases mitochondrial membrane
 128 potential (**Figure 2G**) and impairs P2X7R physiological responses such as motility (**Figure 2E,F**)
 129 and oxygen radical generation (**Figure 2H**). All these responses are restored by supplementation of
 130 methyl-succinate, a Site II electron donor that bypasses Site I, and therefore compensates reduced
 131 NADH dehydrogenase activity.

133

134 **Figure 2** Lack of the P2X7R decreases Complex 1 content of mitochondrial respiratory chain and compromises
 135 physiological responses of cells. . Densitometry (A,C) and Western Blot (B,D) for respiratory chain complexes
 136 (OXPHOS) from HEK293 or HEK293-P2X7R, and N13 WT or N13 R cells is shown. Average absorbance (AU) $\pm$
 137 SEM for HEK293-P2X7 = 0.389 ± 0.0141 , n = 3; HEK293 = 0.1886 ± 0.059 , n = 3; N13 R = 0.301 ± 0.0018 , n = 3;
 138 N13 WT = 0.712 ± 0.039 , n = 3. Ten μ g of protein was loaded in each lane. Scratch wound assay in N13 WT or N13 R
 139 culture (E). Cells were grown in 24-well plates and the wounds were made with a sterilized one-milliliter pipette tip at
 140 the same time in all wells at 80% confluence. Wound width (F) was measured with Image J at time 0 (T0) and after 24
 141 h (T24). Average $\pm$ SEM wound width at T24 as a percentage at T0 was 42.67 ± 5.28 , n = 5, for N13 WT, and $57.11 \pm$
 142 2.269 , n = 5, for N13 R. In the presence of 2.5 mM methylsuccinate wound width at T24 was 35.90 ± 2.39 for N13 R,
 143 and 40.14 ± 5.64 , n = 5, for N13 WT. Mitochondrial potential in the absence or presence of 2.5 mM methyl succinate
 144 was measured by TMRM fluorescence with an epifluorescence microscope (average arbitrary fluorescence units $\pm$
 145 SEM, N13 R basal 1075 ± 16.19 , n = 35, vs N13 R+methylsuccinate 1265 ± 29.35 , n = 35) (G). Oxygen radical
 146 production in the absence or presence of 2.5 mM methyl succinate was measured by 2',7'-dichlorodihydrofluorescein
 147 (DCF) with a Tali image-based cytometer (average fluorescence arbitrary units $\pm$ SEM for N13 WT basal 1795 ± 68.67 ,
 148 n = 438; N13 R basal 1184 ± 34.20 , n = 347); N13 WT + methylsuccinate 2854 ± 111.7 , n = 439; N13 R +
 149 methylsuccinate 1933 ± 41.26 , n = 383) (H), as described in Materials and Methods. P values are calculated with the
 150 two-tailed unpaired Student's t-test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

151 **The P2X7R localizes to the mitochondria.** Previous data hinted to a P2X7R localization to the
 152 nuclear membrane (Atkinson et al. 2002), and possibly to phagosomes (Kuehnel et al. 2009). Due to
 153 its dramatic effect on oxidative phosphorylation, we hypothesized that the P2X7R, besides its
 154 canonical plasma membrane expression, might also localize to the mitochondria. To address this
 155 issue, we probed mitochondrial fractions from cells natively expressing the P2X7R, i.e. MEF WT,
 156 and N13 WT, or transfected with the P2X7R, i.e. HEK293-P2X7R, with an anti-P2X7R antibody
 157 raised against the C-terminal domain. As shown in **Figure 3A-C**, a band of the approximate MW
 158 of 75 kDa is labeled in mitochondrial fractions from the three different cell populations. A band
 159 corresponding to the P2X7 subunit is clearly visible not only in a mitochondrial preparation
 160 obtained according to standard fractionation procedures, but also in a highly purified mitochondrial
 161 fraction (HMF) enriched in mitochondrial calcium uniporter (MCU) content and fully lacking IP3
 162 receptor 3 (IP3R3) and β -tubulin.

164

165 **Figure 3** The P2X7R localizes to the mitochondria. Western blot analysis of lysates (10 μ g of protein/lane) from whole
166 cells (H), a crude mitochondrial fraction (MF) or a highly purified mitochondrial fraction (HMF). Cytosolic fraction (C)
167 was loaded as control. Cell fractionation was performed as described in Materials and Methods. Inositol phosphate
168 receptor 3 (IP3R3), β -tubulin and mitochondrial calcium uniport (MCU) were used as markers of endoplasmic
169 reticulum, cytosol and mitochondria, respectively.

170 To clarify the membrane topology of the P2X7 subunit, this highly purified mitochondrial fraction
171 from HEK293-P2X7R (**Figure 4A,C,E**) and N13 WT (**Figure 4B,D,F**) cells was treated with
172 proteinase K in iso-osmotic or hypo-osmotic medium and stained with antibodies raised against the
173 C- or N-terminal tail, or against the P2X7R extracellular loop. Proteinase K treatment in iso-
174 osmotic medium abrogates staining with the anti-extracellular loop antibody as well as TOM20
175 staining, a transporter localized on the outer mitochondrial membrane, but does not affect P2X7R
176 staining with the anti-C-terminal and the anti-N-terminal antibodies, or with an antibody against
177 TIM23, a transporter localized on the inner mitochondrial membrane. Treatment with proteinase K
178 in hypo-osmotic medium, which causes mitochondrial swelling and thus permeabilizes the outer
179 membrane, decreases but does not abrogate staining with either the N- or the C-terminal antibody.
180 TIM23 labelling is also strongly decreased in hypo-osmotic medium. Finally, membrane
181 solubilization by Triton X-100 treatment, which makes all mitochondrial compartments accessible
182 to proteinase K, abolishes reactivity to all antibodies. These data show that the bulky middle P2X7R
183 domain (the extracellular loop) is exposed on the outer mitochondrial membrane, facing the
184 cytoplasm. However, both the N- and C-termini are largely protected against proteolytic cleavage
185 under hypo-osmotic conditions, which suggests that they might be embedded into the phospholipid
186 bilayer. The P2X7R C-terminus is known to be highly palmitoylated (Gonnord et al. 2009)
187 (Karasawa et al. 2017), a modification that facilitates insertion into the lipid bilayer.

188

189 **Figure 4** The P2X7R localizes to the outer mitochondrial membrane. Western blot analysis of a highly purified
190 mitochondrial fraction from HEK293-P2X7R or N13 WT exposed to proteinase K (PK) (100 µg/ml) in iso- (IsoM) or
191 hypo- (HypoM) osmotic buffer and stained with antibodies raised against the extracellular (A,B), the C-terminal (C,D)
192 or N-terminal (E,F) domains of the P2X7R. H, whole cell lysate; MF, mitochondrial fraction; HMF, highly purified
193 mitochondrial fraction; IsoM+PK, mitochondrial fraction incubated in iso-osmotic medium plus PK; HypoM+PK,
194 mitochondrial fraction incubated in hypo-osmotic medium plus PK. Trit+PK, mitochondrial fraction incubated in
195 Triton-X100-supplemented medium plus PK. Hsp60, TIM23 and TOM20 were used as markers of mitochondrial
196 matrix, inner and outer mitochondrial membrane, respectively.

197 We next investigated mitochondrial P2X7R localization by confocal microscopy. To this aim,
 198 HEK293-P2X7R cells were co-labelled with an anti-P2X7R C-tail antibody and a TOM20 antibody
 199 (**Figure 5A-D**). Under resting conditions, weak P2X7R localization to the mitochondria is detected
 200 (yellow arrows in the inset in panel **A**). Mitochondrial localization is enhanced by stimulation with
 201 the P2X7R agonist benzoyl-ATP (BzATP), or with stressing agents such as rotenone or H₂O₂
 202 (yellow arrows in insets in panels **B-D**, and panels **E-G**). P2X7R recruitment to the mitochondria
 203 starts soon after application of the different stimulants, and reaches a peak after about 1 h, with
 204 BzATP or rotenone, while with H₂O₂ peak increase is much faster (10 min), followed by a decline
 205 after 1 h, and by a delayed increase at 6 h. Total P2X7 subunit cell content does not change in
 206 response to the different stimuli (**Figure 5H**).

207 Lack of P2X7R heavily affects several immune cell pathophysiological responses, very
 208 likely due to a defective energy metabolism (Di Virgilio et al. 2017) (Solle et al. 2001) (Hubert et
 209 al. 2010) (Karmakar et al. 2016) (Borges da Silva et al. 2018). We thus wondered whether P2X7R
 210 lack might cause effects extending beyond immunometabolism, e.g. to other tissues heavily
 211 dependent on oxidative phosphorylation. We thus investigated P2X7R localization and function in
 212 the heart.

Figure 5 Confocal microscopy analysis of P2X7R localization. HEK293-P2X7R cell were plated on sterilized glass coverslips in 24-well plate, at a density of 5,000 cells/well in DMEM-F12 medium, and were left either untreated (**A**), or challenged with BzATP (**B,E**), rotenone (**C,F**) or H₂O₂ (**D, G**). After 1 h (BzATP or rotenone) or 10 min (H₂O₂) cells were fixed and stained with anti TOM20 (green) and anti P2X7R (fuchsia) antibodies. A magnification of the selected region is shown in the inset. Areas of co-localization (yellow arrow) are shown in white. Ten random fields from three independent experiments were analysed. Graphs (**E-G**) report quantitation of P2X7R/mitochondria localization with the Manders colocalization coefficient (expressed as percentage of P2X7R signal overlapping with TOM20 marker). Scale bar = 10 μ m. Total P2X7R content at western blot under the different experimental conditions and at different time points is shown in (**H**). Exact averages $\pm$ SEM for panels **E,F,G** are shown in Supplementary Table II. P values are calculated with the two-tailed unpaired Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

224 **The P2X7R localizes to heart mitochondria and affects heart function.** The P2X7R is expressed
 225 in whole mouse hearts and in a highly purified heart mitochondrial fraction (**Figure 6A**). Hearts
 226 from P2X7R-KO mice lack P2X7R immunoreactivity (**Figure 6B**), do not differ in weight from
 227 those from P2X7R-WT, but are of significantly larger size (**Figure 6C,D**). Morphometric analysis
 228 by electron microscopy revealed that mitochondria from P2X7R-KO versus P2X7R-WT mice are
 229 slightly but significantly smaller (**Figure 6E,G**), although total mass is unchanged based on the
 230 content of the TOM20 and TIM23 markers. To investigate heart function, we analyzed *in vivo*
 231 cardiac performance by High-frequency Ultrasound imaging system (Di Lascio et al. 2014) (**Figure**
 232 **6H**). Left ventricle systolic volume (Vols) is slightly higher in P2X7R-KO mice (**Figure 6I**), while
 233 left ventricle diastolic volume (Vold) is lower (**Figure 6J**), but differences do not reach statistical
 234 significance. More interestingly, four basic heart indexes are significantly lower in P2X7R-KO
 235 versus WT mice: stroke volume (**Figure 6K**), fractional shortening (**Figure 6L**), ejection fraction
 236 (**Figure 6M**), and cardiac output (**Figure 6N**).

Figure 6 Lack of the P2X7R impairs cardiac function. Purified heart mitochondrial fractions (see Materials and

239 Methods) from WT (**A**) or P2X7R-KO (**B**) C57BL/6 mice were analyzed for P2X7R expression by Western Blot.
 240 Hsp60, TIM23 and mitochondrial calcium uptake protein 2 (MICU2) were used as markers of mitochondrial matrix,
 241 inner or outer mitochondrial membrane, respectively. Excised whole hearts from P2X7R WT (average weight $g \pm SEM$
 242 $= 0.1935 \pm 0.005343$, $n = 6$) or P2X7R-KO (average weight $g \pm SEM = 0.1965 \pm 0.01169$, $n = 6$) mice were weighed
 243 (**C**), measured by caliper to assess volume (average volume $mm^3 \pm SEM$ P2X7R WT = 176.5 ± 21.20 , $n = 6$; average
 244 volume $mm^3 \pm SEM$ P2X7R-KO = 261.3 ± 9.229 , $n = 6$) (**D**), and analyzed by TEM (**E-G**). Mitochondrial area (μm^2) is
 245 expressed as average $\pm SEM$ for WT (0.1059 ± 0.002917 , $n = 289$) vs P2X7-KO (0.08657 ± 0.003239 , $n = 253$) (**G**). In
 246 vivo heart indexes were measured with the High-frequency Ultrasound imaging system (see Methods) shown in (**H**).
 247 Vols, left ventricle systolic volume (**I**); Vold, left ventricle diastolic volume (**J**); SV, stroke volume (**K**); FS, fractional
 248 shortening (**L**); EF, ejection fraction (**M**); CO, cardiac output (**N**). Data are presented as median [IQR] of $n = 16$ mice
 249 for condition; *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

250 A decreased cardiac function is anticipated to have a negative impact on physical fitness, therefore
251 we verified whether P2X7R-KO mice had a reduced performance in a Rotarod wheel test. As
252 shown in **Figure 7A**, P2X7R-KO mice spend significantly less time on the wheel compared to
253 P2X7R-WT. Finally we anticipated that body temperature homeostasis might be impaired in
254 P2X7R-KO mice due to mitochondrial dysfunction. This prediction is fulfilled as average resting
255 body temperature measured with an infrared camera is significantly lower in P2X7R-KO versus
256 P2X7R-WT mice (**Figure 7B,C**).

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Figure 7 Lack of the P2X7R reduces physical fitness and surface body temperature. Physical fitness of C57Bl/6 P2X7 WT and KO mice (A) was investigated in a standard Rotarod test by measuring time (seconds) spent on the wheel, as described in Methods. Data are presented as average $\pm$ SEM for WT (803.3 ± 44.74 , $n = 6$) and P2X7R-KO mice (659.3 ± 34.45 , $n = 6$). For body temperature measurement (B,C), WT and P2X7R-KO mice were placed on a warm surface in a 22° C heated room, and body images acquired and measured with a Thermacam P25 infrared Camera. Representative thermal images from one WT (right) and one P2X7R-KO (left) mouse from dorsal or ventral view (B). Each temperature measurement was performed in triplicate in a total of 7 mice for condition. Data are presented as average $^{\circ}\text{C} \pm \text{SEM}$ for WT (33.37 ± 0.2607 , $n = 7$) and P2X7R-KO (34.4 ± 0.226 , $n = 7$) mice. p values are calculated with the two-tailed unpaired Student's t-test. *, $p < 0.05$.

267 Discussion

268 ATP is released into the extracellular space mainly during inflammation and muscle contraction
 269 (Bours et al. 2006) (Forrester 1966). In these conditions, extracellular ATP, acting at plasma
 270 membrane P2YRs and P2XRs, promotes immune cell recruitment, release of inflammatory
 271 cytokines, endothelial cell activation and vasodilatation (Di Virgilio et al. 2017) (Burnstock 2017).
 272 The P2X7R subtype is known for its pro-inflammatory and cytotoxic activity, but paradoxically
 273 also for its trophic effect on energy metabolism when stimulated by low agonist concentrations
 274 (Baricordi et al. 1999). While the mechanism of P2X7R-mediated cytotoxic activity has been
 275 extensively investigated (Di Virgilio et al. 1998), the molecular basis of the trophic effect is as yet
 276 poorly unknown. Several years ago we showed that tonic stimulation of the P2X7R increases the
 277 Ca^{2+} concentration of the mitochondrial matrix, supports mitochondrial metabolism and increases
 278 intracellular ATP content (Adinolfi et al. 2005). These studies complemented earlier findings again
 279 from our laboratory showing that the P2X7R, contrary to the general opinion, is not simply a
 280 cytotoxic receptor but rather a “dual function” receptor, depending on the level of activation and the
 281 metabolic state of the target cell (Baricordi et al. 1999) (Di Virgilio, Ferrari, and Adinolfi 2009).
 282 Trophic, growth-promoting activity of the P2X7R has been now demonstrated in several *in vitro*
 283 and *in vivo* systems (Di Virgilio et al. 2018) (Lara et al. 2020). Furthermore, accruing indirect
 284 evidence supports the view that the P2X7R modulates cell and whole body energy homeostasis
 285 being involved in dysmetabolisms, obesity and hormonal dysfunctions (Giacovazzo et al. 2019)
 286 (Novak and Solini 2018) (Solini and Novak 2019) (Giacovazzo, Apolloni, and Coccurello 2018). A
 287 cardio-protective activity for the P2X7R has also been proposed (Vessey, Li, and Kelley 2010).

288 Participation in energy homeostasis is intriguing since the only (so far) *bona fide*
 289 physiological agonist of the P2X7R is ATP, i.e. the universal fundamental intermediate in
 290 biological energy transactions. Ample evidence shows that ATP is released via non-lytic
 291 mechanisms and accumulates into the extracellular space at sites of trauma or inflammation (Di
 292 Virgilio, Sarti, and Coutinho-Silva 2020). The P2X7R itself has a leading role in setting

extracellular ATP levels since it has been shown that this receptor may serve as a conduit for ATP release (Pellegatti et al. 2005) (Suadicani, Brosnan, and Scemes 2006). This is not surprising as the P2X7R macropore has a molecular cut-off of about 900 D, sufficient to allow permeation of a highly hydrophilic molecule of molecular mass of 507 such as ATP. Thus, it appears that the P2X7R has a very intricate relationship with its own agonist because on one hand it promotes ATP synthesis and transport across the plasma membrane, and on the other is a target of ATP. This intimate reciprocal relationship seems very logical in light of the pathophysiological implications of ATP accumulation into the extracellular space under a variety of conditions requiring an active energy metabolism. For example, ATP accumulates into the interstitium of exercising muscles (Forrester 1966), therefore stimulation of muscle cell P2X7R may support muscle metabolism under stress. Even more interesting in light of its role in inflammation, is P2X7R ability to “sense” ATP levels at inflammatory sites and promote a whole range of defensive responses requiring an increased energy metabolism, such as chemotaxis, phagocytosis, release of cytolytic granules, generation of reactive oxygen species (Di Virgilio et al. 2017). In this regard, the well-known low affinity of the P2X7R for ATP is of advantage because the intracellular ATP-synthetic machinery will be stimulated only when a large amount of ATP accumulates into tissue interstitium, i.e. under stress conditions when intracellular energy stores are depleted and a boost to energy synthesis is needed.

Controlled activation of the P2X7R might in principle support mitochondrial ATP synthesis in different ways, e.g. by increasing fatty acid oxidation (Giacovazzo, Apolloni, and Coccurello 2018), by facilitating glucose uptake and consumption (Glas et al. 2009), or by increasing mitochondrial Ca^{2+} levels and thus stimulating NADH oxidase activity (Griffiths and Rutter 2009). We previously hypothesized that basal activation of the P2X7R might increase basal cytoplasmic Ca^{2+} levels, and thus raise mitochondrial matrix Ca^{2+} . This prediction is confirmed by experiments reported in the present work showing that P2X7R-deficient cells have a significantly lower mitochondrial Ca^{2+} concentration versus P2X7R-proficient cells (see also (Adinolfi et al. 2005)).

319 Since mitochondrial NADH oxidase is known to be stimulated by Ca^{2+} , the controlled increase in
 320 matrix Ca^{2+} is reasonably anticipated to enhance ATP synthesis (Griffiths and Rutter 2009). Thus,
 321 the P2X7R indirectly supports oxidative phosphorylation by modulating mitochondrial Ca^{2+} levels.
 322 However, NADH oxidase expression levels are also reduced in P2X7R-deficient cells. Thus, the
 323 P2X7R affects NADH oxidase in a dual fashion: by increasing its expression as well as its activity.
 324 Complex II expression is also affected by lack of P2X7R, but in opposite ways in HEK293 versus
 325 N13 R cells. In HEK293 cells, transfection with the P2X7R down-modulates Complex II
 326 expression, while in N13 R versus N13 WT cells Complex II expression is unchanged or at best
 327 slightly decreased. Down-modulation of Complex II in HEK293-P2X7R cells might be a side effect
 328 of forced P2X7R overexpression since it is not observed in cells natively expressing the P2X7R. A
 329 major role of NADH oxidase defective activity as a cause of reduced mitochondrial metabolic
 330 efficiency in P2X7R-less cells is supported by the observation that supplementation of
 331 methylsuccinate, a membrane-permeant substrate that bypasses Site I of the respiratory chain and
 332 feeds directly into Site II, restores near normal mitochondrial membrane potential.

333 Effect of P2X7R on NADH oxidase might be simply indirect, but based on the present data,
 334 might also be directly mediated by P2X7R mitochondrial localization, and therefore be more
 335 intimately involved in the modulation of mitochondrial physiology. Although the P2X7R is
 336 generally considered a plasma membrane channel, previous anecdotal evidence shows that it also
 337 localizes to phagosomes (Kuehnelt et al. 2009), and the nuclear membrane (Atkinson et al. 2002).
 338 The physiological function of intracellular P2X7R has never been investigated, with the exception
 339 of the phagosomal localization which has been implicated in killing of phagocytosed pathogens by
 340 facilitating fusion of early phagosomes with lysosomes (Fairbairn et al. 2001). The discovery that
 341 the P2X7R is present on the mitochondria adds further complexity to the intracellular physiology of
 342 P2X7R.

343 Localization of the P2X7 subunit to the mitochondria in the present work was probed with
 344 three different antibodies raised against the N-terminal, the central region (extracellular loop) and

the C-terminal domain of the receptor. We used both a mitochondrial fraction obtained with the standard fractionation procedure, and a highly purified mitochondrial fraction, to avoid always possible sources of contamination by intracellular organelles or the plasma membrane. Use of antibodies raised against different domains of the receptor revealed that the P2X7 subunit localizes to the outer mitochondrial membrane and allowed a tentative resolution of the membrane topology. Abolition of staining with the anti-middle domain antibody by proteinase K treatment under isosmotic conditions suggests that the middle bulky domain faces the cytoplasm, while the N- and C-termini face the inter-membrane space. Under hypo-osmotic conditions, the N- and C-termini are still partially protected from proteolysis, an effect that might be due to the tight association of these two residues with phospholipid bilayer (Gonnord et al. 2009). This membrane topology raises several intriguing questions.

The P2X7R is a cation-selective, ATP-activated channel that when expressed on the plasma membrane faces the extracellular space with the bulky, central, domain, while N- and C-termini face the cytoplasm (North and Surprenant 2000) (Karasawa and Kawate 2016). The middle domain also contains the ATP-binding sites. Therefore, ATP-binding sites in mitochondrial P2X7R face the ATP-rich cell cytoplasm. Assuming that P2X7 monomers assemble on the outer mitochondrial membrane to form a functioning P2X7R, the receptor should be constitutively activated by cytoplasmic ATP, and therefore constantly short-circuit cations across the outer mitochondrial membrane. This should not substantially alter mitochondrial energetics since it is well known that the outer mitochondrial membrane is freely permeable to small ions, but raises the question of the physiological meaning of such a pathway. On the other hand, it might well be that P2X7 subunits do not assemble into a functioning receptor, but again the meaning of these hypothetically “silent” monomers is unknown. Our data show that P2X7R expression besides improving mitochondrial metabolism also increases mitochondria size and thickness of the mitochondrial network (see also (Adinolfi et al. 2005)). These effects might be unrelated to P2X7R channel function and rather be dependent on a specific (but as yet unknown) structural activity of the isolated subunits.

371 Although several mechanistic details are still missing, the effect of P2X7R depletion on *in*
 372 *vivo* performance is dramatic. We investigated the effect of P2X7R depletion in cardiac function, as
 373 the heart is heavily dependent on mitochondrial metabolism. Cardiac mitochondria are smaller
 374 while heart volume is larger in P2X7R deficient versus proficient mice. More importantly, basic
 375 cardiac parameters (stroke volume, ejection fraction, cardiac output and fractional shortening) and
 376 physical performance (Rotarod test) are significantly lower in the absence of the P2X7R.
 377 Interestingly, surface body temperature is also lower in the P2X7R-deleted mice. Cardiac indexes in
 378 P2X7R-deleted mice are highly reminiscent of the clinical findings observed in patients affected by
 379 dilated cardiomyopathy. This is not surprising as dilated cardiomyopathy is a typical manifestation
 380 of mitochondrial disease (Meyers, Basha, and Koenig 2013).

381 Involvement of the P2X7R in heart dysfunction is at present very speculative. A loss-of-
 382 function mutation in the *P2RX7* gene (556C>A) has been recently found to associate with
 383 hypertrophic cardiomyopathy in a family from India (Biswas et al. 2019), but another study found
 384 no association between two *P2RX7* SNPs, the gain of function 489C>T and the loss-of-function
 385 1513A>C, and acute heart failure in a geriatric population (Pasqualetti et al. 2017). Despite this
 386 contrasting evidence, our study highlights an as yet un-described function of the P2X7R in the
 387 regulation of mitochondrial metabolism and points to a potentially relevant role in cardiac function
 388 and physical fitness.

Materials and Methods

Ethical compliance. All mouse studies were performed under guidance of the University of Ferrara Institutional Animal Welfare and Use Committee and the Ethical Panel of the University of Pisa (approved protocol n. 943/2015-PR), in accordance with Italian regulatory guidelines and laws (D.Lvo 26/2014, Ministry of Health authorizations n. 75/2013-B and 76/2013-B), and the European Directive (2010/63/UE).

Reagents. Benzoyl ATP (BzATP, cat. n. B6396), rotenone (cat. n. R8875), hydrogen peroxide solution (H₂O₂, cat. n. 16911) and methyl-succinate (cat n. M81101) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylrhodamine methyl ester (TMRM, cat n. T668, Molecular Probes, Leiden, The Netherlands) was dissolved in DMSO to obtain a 10 mM stock solution and then diluted in the appropriate buffer. Carbonyl cyanide *α*-[3-(2-benzothiazolyl)6-[2-[2-[bis(carboxymethyl)amino]-5-methylphenoxy]-2-oxo-2H-1-benzopyran-7yl]-*b*-(carboxymethyl)-tetrapotassium salt (FCCP, cat. n. C2920, Sigma-Aldrich) was solubilized in ethanol to a final stock concentration of 10 mM. For Seahorse analysis, a Seahorse XF Cell Mito Stress Test Kit was used including the following compounds: oligomycin (stock solution 100 μM), FCCP (stock solution 100 μM), and a mix of rotenone/antimycin A (stock solution 50 μM) (cat n. 103015-100, Agilent, Santa Clara, USA). Crystal violet was purchased from Sigma-Aldrich (cat n. C0775) and used as a 0.1% solution in 10% ethanol. Krebs-Ringer bicarbonate solution (KRB) contained 125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM Na₂HPO₄, 5.5 mM glucose, 20 mM NaHCO₃, 2 mM l-glutamine and 20 mM HEPES (pH 7.4), and was supplemented with 1 mM CaCl₂.

Cell culture and transfections. HEK293 cells were cultured in DMEM-F12 (cat n. D6421, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, cat n. 16000044), 100 U/ml penicillin, and 100 mg/ml streptomycin (cat n. 15140130) (all from Invitrogen, San Giuliano Milanese, Italy). Stable P2X7R-transfected clones were kept in the continuous presence of 0.2

415 mg/ml G418 sulphate (Geneticin, cat. n. 509290, Calbiochem, La Jolla, CA). Experiments, unless
 416 otherwise indicated, were performed in the following saline solution: 125 mM NaCl, 5 mM KCl, 1
 417 mM MgSO₄, 1 mM NaH₂PO₄, 20 mM HEPES, 5.5 mM glucose, 5 mM NaHCO₃, and 1 mM CaCl₂,
 418 pH 7.4. N13 microglial cells, wild-type (N13 WT) and ATP-resistant (N13 R), were cultured in
 419 RPMI 1640 medium, (cat n. R0883, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10%
 420 heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Primary mouse microglia
 421 cells were isolated from 2 to 4-day-old post-natal mice as described previously (Sanz and Di
 422 Virgilio 2000). More than 98% of cells were identified as microglia using a macrophage cell-
 423 specific F4/80 rabbit mAb antibody (cat. n. MCA497, Serotec, Dusseldorf, Germany) followed by
 424 staining with Oregon Green 488 goat anti-rabbit IgG (cat. n. O-11038, Molecular Probes, Leiden,
 425 The Netherlands). Microglia cells were plated in astrocyte-conditioned high glucose-DMEM
 426 medium (cat n. 10566016), supplemented with 2 mM glutaMAX™ (Gibco Life Technologies
 427 Europe BV, Monza, Italy), 10% heat-inactivated FCS, 100 U/mL penicillin and 100 mg/mL
 428 streptomycin, and used for experiments 24 h after plating (Sanz and Di Virgilio 2000). MEFs were
 429 isolated from pregnant mice at 13 or 14 day post coitum. Mice were sacrificed by cervical
 430 dislocation, embryos were harvested, and MEFs were isolated as previously described (Missiroli et
 431 al. 2016) and cultured in DMEM medium supplemented with 10% FBS, penicillin/streptomycin,
 432 and 2 mM L-glutamine.

433

434 **Seahorse analysis.** Oxygen consumption in cell lines (N13 WT, N13 R, HEK293 WT and
 435 HEK293-P2X7R) and primary cells (microglia and MEFs) was measured using the Seahorse
 436 Bioscience XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent, Santa Clara CA,
 437 USA). Cells were seeded in triplicate in XF96 96-well cell culture plate (Seahorse Bioscience) in a
 438 volume of 80 µl/well in DMEM complete medium at a density of 15,000/well (HEK293),
 439 20,000/well (microglia and MEF), or 22,000/well (N13 WT and N13 R). The XF96 sensor cartridge
 440 was hydrated with 200 µl/well of XF Calibrant buffer and placed overnight in a 37°C incubator

441 without CO₂. On day two, incubation medium was replaced just before running the assay with
442 Seahorse assay medium supplemented with glucose (10 mM) and sodium pyruvate (2 mM), pH 7.4,
443 and equilibrated for 1 h at 37 °C in the absence of CO₂. Oligomycin, to inhibit ATP synthesis,
444 FCCP, to uncouple oxidative phosphorylation, and a mix of rotenone/Antimycin A, to block
445 electron transport, were sequentially added at various times. Oxygen consumption rates (OCRs)
446 were measured before and after injection of the different inhibitors. OCRs were normalized to cell
447 content in each well determined by crystal violet staining.

448

449 **Measurement of mitochondrial membrane potential and Ca²⁺ concentration.** Mitochondrial
450 membrane potential ($\Delta\Psi_m$) was measured by confocal microscopy with TMRM (10 nM), at an
451 emission wavelength of 570 nm. FCCP was used to collapses $\Delta\Psi_m$. Mitochondrial Ca²⁺
452 concentration was measured with the last-generation GCaMP probe targeted to the mitochondrial
453 matrix (Chen et al. 2013). We chose the GCaMP6m version due to its high Ca²⁺ affinity (K_d of 167
454 nM). HEK293 and HEK293-P2X7 cells were grown on 24-mm coverslips, transfected with
455 mtGCaMP6m-encoding plasmid and imaged with an IX-81 automated epifluorescence microscope
456 (Olympus Italy, Segrate, Italy) equipped with a 40X oil immersion objective (numerical aperture
457 1.35) and an ORCA-R2 charge-coupled device camera (Hamamatsu Photonics, Hamamatsu, Japan).
458 Excitation wavelengths were 494/406 nm and emission 510 nm. The 496/406 ratio is proportional
459 to the Ca²⁺ concentration and independent of probe expression level. Analysis was performed with
460 ImageJ.

461

462 **NADH measurement.** NADH autofluorescence was measured using an inverted epifluorescence
463 microscope equipped with a 40X oil-fluorite objective. Excitation at a wavelength of 360 nm was
464 provided by a xenon arc lamp, with the beam passing through a monochromator (Cairn Research,
465 Graveney Road, Faversham, UK). Emitted light was reflected through a 455 nm long-pass filter to a
466 cooled Retiga QImaging CCD camera (Cairn Research) and digitized to 12 bit resolution. Imaging

data were collected and analyzed with ImageJ. Alternatively, NADH levels in N13 WT and N13 R cells were measured using a NAD/NADH assay kit (NAD/NADH Assay Kit, Colorimetric, cat n. ab65348, Abcam, San Francisco, CA, USA), according to manufacturer's indications.

Subcellular Fractionation. Cells were harvested, rinsed in PBS by centrifugation at 500 g for 5 min, re-suspended in homogenization buffer (225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl, 0.1 mM EGTA, and phenylmethylsulphonyl fluoride, PMSF, pH 7.4) and gently disrupted by dounce homogenization. The homogenate was centrifuged twice at 600 X g for 5min to remove nuclei and unbroken cells, and the supernatant centrifuged at 10,300 X g for 10 min to pellet crude mitochondria. The supernatant was further centrifuged at 100,000 X g for 90 min in a 70-Ti rotor (Beckman Coulter, Indianapolis, IN, USA) at 4 °C to pellet the endoplasmic reticulum (ER) fraction. The crude mitochondrial fraction, re-suspended in isolation buffer (250 mM mannitol, 5 mM HEPES, 0.5 mM EGTA, pH 7.4), was centrifuged through Percoll gradient (Percoll medium: 225 mM mannitol, 25 mM HEPES pH 7.4, 1 mM EGTA and 30% v/v Percoll, cat n. GE17-0891-01, Sigma-Aldrich) in a 10- ml polycarbonate ultracentrifuge tube. After centrifugation at 95,000 X g for 30 min a dense band containing purified mitochondria was recovered near the bottom of the gradient and further processed as described (Wieckowski et al. 2009). Purity of the preparation was assessed by Western blot analysis of ER and mitochondrial markers. For mitochondrial fractionation, a total of at least 600 mg of isolated mitochondria was resuspended in different buffers, i.e. isosmotic buffer (10 mM Tris-MOPS, 0.5 mM EGTA-Tris, 0.2 M Sucrose, pH 7.4) with or without proteinase K (100 µg/ml), hyposmotic buffer (10 mM Tris-MOPS, 0.5 mM EGTA-Tris pH 7.4) plus proteinase K (100 µg/ml) and hyposmotic buffer plus proteinase K (100 µg/ml) and Triton (0.01% v/v Triton X-100). Samples were incubated on ice for 30 min with gentle mixing. After this time, 2 mM PMSF was added and the samples were incubated for an additional 5 min at 4°C. Proteins were precipitated with TCA/Acetone, 10% trichloroacetic acid in acetone (w/v) in a 1:6 ratio, precipitated at 4 °C for 30 min, and centrifuged at 14,000 X g for 30 min at 4

493 °C. Supernatant was discarded and the pellet was washed with 500 µl ice-cold acetone. Samples
494 were centrifuge at 14,000 g for 15 min at 4 °C, resuspended in Laemmli sample buffer, and
495 separated by SDS-PAGE.

496

497 **Isolation of mitochondria from mouse heart.** Mouse hearts were washed twice with ice-cold PBS
498 buffer to remove blood, minced, and finally resuspended in buffer A (180 mM KCl, 10 mM EDTA,
499 20 mM Tris HCl pH 7.4), plus 0.5 mg/ml trypsin. Samples were then incubated for 30 min at 4° C
500 under magnetic stirring. At the end of this incubation, they were re-suspended in buffer B (180 mM
501 KCl, 20 mM Tris HCl and 0.05 mg/ml albumin, pH 7.4). The homogenate was centrifuged twice at
502 4 °C for 2 min at 600 X g, the supernatant collected and further centrifuged at 4 °C for 10 min at
503 25,000 X g. The pellet (crude mitochondria) was re-suspended in isolation buffer and processed as
504 describe in “Subcellular fractionation” to obtain pure mitochondria.

505

506 **Calculation of heart volume and weight.** Hearts from 8-week-old C57BL/6-WT or C57BL/6-
507 P2X7 KO mice were isolated, washed in cold PBS, weighted and measured with a caliper. The
508 following formula was used to calculate volume: $\text{volume} = \pi/6 \times \text{length} \times \text{width}^2$

509

510 **Histology.** Samples were reduced and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH
511 7.4, and post-fixed in 2% osmium tetroxide in the same buffer, dehydrated by increasing passages
512 in acetone and included in Araldite Durcupan ACM (Fluka address). Semi-thin sections were
513 prepared with a Reichert Ultracut S ultramicrotome (Leica Microsystems, Buccinasco, Italy),
514 stained with a 1% aqueous solution of toluidine blue, and observed with a light Nikon Eclipse E800
515 microscope (Nikon Corporation, Tokyo, Japan). Ultrathin sections were prepared with an
516 ultramicrotome the Reichert Ultracut S ultramicrotome, counterstained with uranyl acetate in
517 saturated solution and lead citrate (Reynolds 1963), and observed with a transmission electron
518 microscope Hitachi H800 at 100 Kv (Hitachi High Technologies Corporation, Brughiero, Italy).

519

520 **Measurement of reactive oxygen species.** The fluorogenic substrate 2',7'-dichlorofluorescein
521 diacetate was used to measure reactive oxygen species (ROS). Fluorescence intensity was
522 measured with an image-based cytometer (Tali™ Image-based Cytometer, Invitrogen). Cells were
523 pipetted into a Tali Cellular Analysis Slide and loaded into the cytometer. Bright field and green
524 fluorescence images were captured and analysed with specific assay algorithms. Histograms are
525 then generated to display cell size and fluorescence intensity.

526

527 **Western blot.** Total cell lysates were prepared in RIPA buffer, 50 mM Tris-HCl pH 7.8, 150 mM
528 NaCl, 1% IGEPAL CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM
529 dithiothreitol, supplemented with protease and phosphatase inhibitors (Inhibitor Cocktail cat. n.
530 P8340, Sigma- Aldrich). Protein concentration was quantified with the Bradford assay (cat n.
531 5000001, Bio-Rad Laboratories, Segrate, Italy). Proteins, 15 µg/lane, were separated by SDS-
532 PAGE, transferred to nitrocellulose membranes, and probed with the different antibodies used at the
533 following dilutions: anti-P2X7 C-terminal, 1:500 (cat. n. APR-004, Alomone, Jerusalem, Israel);
534 anti-P2X7 N-terminal 1:500 (cat. n. SAB2501287, Sigma-Aldrich); anti-P2X7 extracellular loop
535 1:200 (cat. n. P9122, Sigma-Aldrich) anti-TOM 20, 1:1000 (cat. n. D8T4N, Cell Signalling,
536 Leiden, The Netherlands); anti-Hsp60, 1:1000 (cat.n. sc-376240, Santa Cruz); anti-β-Tubulin,
537 1:1000 (cat.n. T5201, Sigma-Aldrich); anti-myosin IIB 1:1000 (cat.n. M7939, Sigma-Aldrich); anti-
538 TIM 23, 1:2000 (cat. n. ab230253 Abcam); anti-actin, 1:5000 (cat. n. A5441, Sigma-Aldrich).
539 Densitometric analysis was performed with the ImageJ software.

540

541 **Immunofluorescence.** HEK293-P2X7R cells were fixed in 4% paraformaldehyde in PBS for 15
542 min, washed three times with PBS, permeabilized for 10 min with 0.1% Triton X-100 in PBS and
543 blocked in 2% BSA-containing PBS for 20 min. Cells were then incubated overnight at 4°C in a
544 wet chamber with the following antibodies: anti-P2X7 (cat. n. P8232, Sigma- Aldrich) and anti-

545 Tom 20 (cat n. 43406, Cell Signalling) diluted 1:100 in 2% BSA-containing PBS. Staining was then
546 carried out with anti-rabbit Alexa 546 (Thermo Fisher Scientific Italy, Monza, Italy, cat n. A-
547 11010) for P2X7 receptor, or with anti-mouse Alexa 633 for TOM 20 (cat n. A-21052). Cells were
548 then washed three times with 0.1% Triton X-100 in PBS. Samples were mounted in ProLong Gold
549 antifade (Invitrogen) and images captured with a confocal microscope (LSM 510, Carl Zeiss,
550 Arese, Italy).

551

552 **Wound healing assay.** N13 WT and N13 R cells were grown to confluence in 24-well plates,
553 RPMI medium was replaced with low serum RPMI medium to slow down proliferation, and the
554 wounds were made simultaneously in all wells with a sterilized pipette tip. Phase contrast pictures
555 were taken at 0 and 24 hours. Cell migration was observed in control condition and after treatment
556 with 2.5 mM methyl succinate. The open wound area at time 0 hours was set at 100%. Images were
557 analysed with an open source ImageJ software.

558

559 **Infrared Thermal Imaging.** Infrared thermal imaging was performed using a thermo-electrically
560 cooled Thermacam P25 camera (Flir Systems Inc., Wilsonville, OR, USA) equipped with a scanner
561 and 24° X 18° lens which detects a 7.5-13 μ M spectral response, as previously reported (Rossato et
562 al. 2014), and an internal calibration system with an accuracy of 0.04 °C. The focal distance was
563 30 cm. Images were captured and analyzed using the Flir quick report software according to
564 manufacturer's specifications. Each mouse (anesthetized with 2.5% isofluorane) was placed on a
565 table at a fixed distance away the camera and images were captured in triplicate.

566

567 **High-frequency Ultrasound examination of mouse heart function.** Sixteen WT and sixteen
568 P2X7-KO male mice were examined with a high-resolution US imaging system (Vevo 2100,
569 FUJIFILM VisualSonics Inc, Toronto, Canada) at 11-12 weeks of age. Animals were anesthetized
570 with isofluorane in an induction chamber connected with a scavenger canister. After induction, each

571 mouse was placed on a temperature-controlled board and the four limbs were coated with
572 conductive paste (Signa Cream, Parker Laboratories Inc, Fairfield, Connecticut, USA) and taped
573 with ECG electrodes. A nose cone was used to keep mice under gaseous anesthesia (1.5%
574 isoflurane in 1 L/min of pure oxygen) during examination, and heart rate (HR), respiration rate
575 (RR) and body temperature (T) were monitored and acquired using the Advancing Physiological
576 Monitoring Unit attached to imaging station. The abdomen was shaved with depilatory cream (Nair,
577 Church & Dwight Canada Corp., Mississauga, ON, Canada) and coated with acoustic coupling gel
578 (SonoSite Cogel, Comedical Sas, Trento, Italy). For acquisition, a 40 MHz US probe (MS550D,
579 FUJIFILM VisualSonics) held in position by a mechanical arm was employed. Images of the heart
580 were acquired using the B-mode modality in parasternal long axis (PLAX) view, and then analysed
581 offline to assess cardiac structure and function (VevoLAB, FUJIFILM VisualSonics Inc, Toronto,
582 Canada). Systolic and diastolic volumes (Vols and Vold), stroke volume (SV), ejection fraction
583 (EF), longitudinal fractional shortening (FS) and cardiac output (CO) were evaluated using the LV
584 Trace analysis tool (Di Lascio et al. 2014) (Gao et al. 2011).

585
586 **Rotarod.** The rotarod test provides information on motor parameters such as coordination, gait,
587 balance, motivation to run and muscle tone (Rozas and Labandeira Garcia 1997). The fixed-speed
588 rotarod test was performed as previously described (Viaro et al. 2008). Animals (six C57BL/6 WT
589 and six C57BL/6-P2X7KO) were placed on a rotating cylinder of 8 cm in a stepwise mode at
590 increasing speeds (from 5 to 45 rpm; 180 s each) and latency to fall was recorded. Three
591 consecutive sessions were run. Prior to testing, animals were trained for 3 consecutive days. The cut
592 off time was set to 30 min and the total time spent on the rod was calculated.

593
594 **Statistical analyses of biochemical and Rotarod data.** All data are shown as mean $\pm$ SEM.
595 Statistical significance was calculated assuming equal SD and variance, with a two-tailed Student's

596 t-test performed with the GraphPad Prism software. A p-value ≤ 0.05 was considered significant.

597 Exact p values for each experiment were calculated and reported in the figure legends.

598

599 **Statistical analysis of cardiac parameters.** The sample size was fixed taking CO as test parameter
600 to allow for higher variability due to combination of geometrical measurements and HR
601 assessments. Considering a standard deviation for CO measure of about 25% and a difference
602 between wt and P2X7 KO mice of about 30% (power $\geq 80\%$ and alpha equal to 0.05, test significant
603 for $p < 0.05$), a sample size of 10 was obtained. All data are presented as median and interquartile
604 range [25-75th IQR]. Two-sided Mann-Whitney test for independent samples was used to highlight
605 differences between WT and P2X7 KO animals. Tests were considered statistically significant with
606 $p < 0.05$.

607

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775

776 **Author contribution**

777 ACS participated in study design, performed most of the experiments, analysed data and revised the
778 manuscript. VVP, SF, SM, ALG, MB participated in most of the experiments and revised the
779 manuscript. PB was responsible for electron microscopy. FF, NDL and CK performed mouse
780 ultrasound examination of mice hearts. AS participated in data analysis and interpretation, and
781 revised the manuscript. SN and MM performed Rotarod tests and revised the manuscript. MR
782 performed infrared thermal imaging. MRW participated in Seahorse analysis and helped in
783 fractionation studies. CG and PP participated in study design and in data analysis, and revised the
784 manuscript. FDV conceived the study, designed most of the experiments, analysed data and wrote
785 the manuscript.

786

787 **Competing interests**

788 FDV is a member of the Scientific Advisory Board of Biosceptre Ltd, a UK-based Biotech involved
789 in the development of P2X7-targeted therapeutic antibodies. Other Authors declare do competing
790 interest.

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792 **Data availability**

793 All data generated or analysed during this study are included in this published article and its
794 supplementary information files, or are available from the corresponding author upon reasonable
795 request.

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818 **Supplementary Table I**

	MEF WT	MEF P2X7-KO
Basal Respiration	376,0 ± 25,77 N=3	258,7 ± 33,33 N=3
ATP Production	302,7 ± 28,10 N=3	195,0 ± 25,94 N=3
Maximal Respiratory Rate	769,3 ± 104,9 N=3	305,7 ± 37,17 N=3
Spare Respiratory Capacity	434,7 ± 89,89 N=3	82,67 ± 3,180 N=3
Uncoupling Level	68,67 ± 7,172 N=3	63,67 ± 10,84 N=3

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	N13 WT	N13 R
Basal Respiration	938,7 ± 82,60 N=3	143,3 ± 48,46 N=3
ATP Production	816,7 ± 59,89 N=3	313,0 ± 114,6 N=3
Maximal Respiratory Rate	4025 ± 300,9 N=3	1565 ± 243,7 N=3
Spare Respiratory Capacity	3087 ± 318,2 N=3	1421 ± 212,8 N=3
Uncoupling Level	221,3 ± 13,87 N=3	171,0 ± 84,54 N=3

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	Microglia WT	Microglia P2X7KO
Basal Respiration	616,3 ± 96,67 N=3	248,3 ± 4,096 N=3
ATP Production	597,0 ± 72,11 N=3	239,0 ± 7,234 N=3
Maximal Respiratory Rate	1663 ± 257,5 N=3	435,3 ± 14,62 N=3
Spare Respiratory Capacity	1381 ± 118,6 N=3	188,0 ± 10,26 N=3
Uncoupling Level	57,00 ± 1,155 N=3	9,333 ± 3,383 N=3

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	HEK293-P2X7	HEK293
Basal Respiration	107,9 ± 5,974 N=6	78,57 ± 3,205 N=7
ATP Production	73,81 ± 5,487 N=6	51,30 ± 2,350 N=7
Maximal Respiratory Rate	156,7 ± 11,76 N=6	93,69 ± 3,845 N=7
Spare Respiratory Capacity	48,78 ± 8,730 N=6	15,12 ± 4,040 N=7
Uncoupling Level	34,10 ± 0,8806 N=6	27,27 ± 1,630 N=7

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823 **Supplementary Table II**

	CTRL	BzATP
CTRL vs. BZATP 10 min	0,7487 ± 0,02080 N=7	0,8478 ± 0,01876 N=10
CTRL vs. BZATP 1 H	0,7487 ± 0,02080 N=7	0,9293 ± 0,02230 N=8
CTRL vs. BZATP 6H	0,7487 ± 0,02080 N=7	0,8801 ± 0,02092 N=11

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	CTRL	Rotenone
CTRL vs. Rotenone 10 min	0,7487 ± 0,02080 N=7	0,7877 ± 0,02371 N=7
CTRL vs. Rotenone 1 H	0,7487 ± 0,02080 N=7	0,8836 ± 0,008011 N=7
CTRL vs. Rotenone 6H	0,7487 ± 0,02080 N=7	0,7639 ± 0,05414 N=7

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	CTRL	H₂O₂
CTRL vs. H ₂ O ₂ 10 min	0,7487 ± 0,02080 N=7	0,8720 ± 0,01481 N=8
CTRL vs. H ₂ O ₂ 1 H	0,7487 ± 0,02080 N=7	0,7936 ± 0,02162 N=7
CTRL vs. H ₂ O ₂ 6H	0,7487 ± 0,02080 N=7	0,8716 ± 0,01225 N=7

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828 **Figure 1-Figure supplement 1.** Lack of the P2X7R doesn't affect mitochondrial cell content.

829 Western blot analysis (a) and densitometry (b) of TOM20, TIM23 and Hsp60 content of N13 WT

830 or N13 R, and MEF WT or MEF P2X7-KO cells.