Supplementary Information

Supplementary Information 1. Functional $P2X_4R$ is expressed in hyperactive microglia in primary culture. In order to determine whether $P2X_4Rs$ are functional in microglia with the hyperactive phenotype, we studied microglia in primary culture (Nakajima, K. et al. Identification of elastase as a secretory protease from cultured rat microglia. *J Neurochem* **58**, 1401-1408, 1992) where all of the cells express high levels of OX42 (Supplementary Figure a). We tested for expression of mRNA encoding P2XRs by using reverse transcriptase-polymerase chain reaction (RT-PCR) with primer pairs specific for each of the 7 subtypes of P2XR ($P2X_1R$-$P2X_7R$). RT-PCR reaction product was detected for $P2X_4R$ as well as for $P2X_7R$, a P2XR subtype previously reported to be expressed in microglia (Ferrari, D. et al. Mouse microglial cells express a plasma membrane pore gated by extracellular ATP. *J Immunol* **156**, 1531-1539, 1996), whereas no RT-PCR product was detected for the other P2XR subtypes. To determine whether $P2X_4R$ protein was expressed, we used immunocytochemistry and observed intense immunofluorescence for $P2X_4R$ (Supplementary Figure b). Thus, microglia in culture express both mRNA and protein for $P2X_4R$. As $P2X_4Rs$ are reported to be highly permeable to Ca^{2+} (Khakh, B. S. et al. International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol Rev* **53**, 107-118, 2001), we investigated whether the $P2X_4Rs$ on these microglia were functional by applying the agonist ATP and monitoring the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in individual cells using the Ca^{2+}-sensitive fluorescent dye fura-2. We found that ATP (50 μM, 10 s) produced a transient increase in the 340/360 emission ratio for fura-2 (n=28 cells), indicating that ATP caused an increase $[Ca^{2+}]_i$ in the microglia (Supplementary Figure c). When the extracellular solution had no added Ca^{2+} the increase in 340/360 emission ratio evoked by ATP was greatly blunted (n=28 cells, $P<0.001$). The ATP-evoked increase in 340/360 ratio was suppressed by

TNP-ATP (n=14 cells, $P<0.01$) but was unaffected by PPADS (n=26 cells, Supplementary Figure d). In addition, the effect of ATP (50 μM) was not altered by brilliant blue G (BBG, 100 nM, n=21 cells), which is known to differentially block $P2X_7R$ but not other subtypes of P2XR (Jiang, L. H., Mackenzie, A. B., North, R. A. & Surprenant, A. Brilliant blue G selectively blocks ATP-gated rat $P2X_7$ receptors. *Mol Pharmacol* **58**, 82-88, 2000). Together, these results indicate that microglia in primary culture, which like hyperactive microglia *in situ* show high levels of OX42, express functional $P2X_4Rs$.

Supplementary Information 2. ATP levels in the cerebrospinal fluid (CSF) from the lumbar spinal cord is not changed following nerve injury. The ATP levels in the CSF collected from the lumbar spinal cord were measured by a luciferin-luciferase bioluminescence assay. We found that the level of ATP in CSF was not different in nerve-injured rats (54.3 ± 14.8 nM, n=4 animals) as compared with naïve controls (58.1 ± 12.4 nM, n=4 animals). Inasmuch as the lack of change in the CSF ATP level indicates that extracellular ATP levels at sites of action at $P2X_4Rs$ on microglia within the dorsal horn are unchanged after nerve injury, we may infer that tactile allodynia following nerve injury depends upon the enhanced expression of $P2X_4Rs$ which are then activated by the constitutive level of the endogenous ligand ATP.