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Supplemental Information

P2Y4 Nucleotide Receptor in Neuronal Precursors Induces Glutamatergic Subtype Markers in Their Descendant Neurons

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Supplemental Information

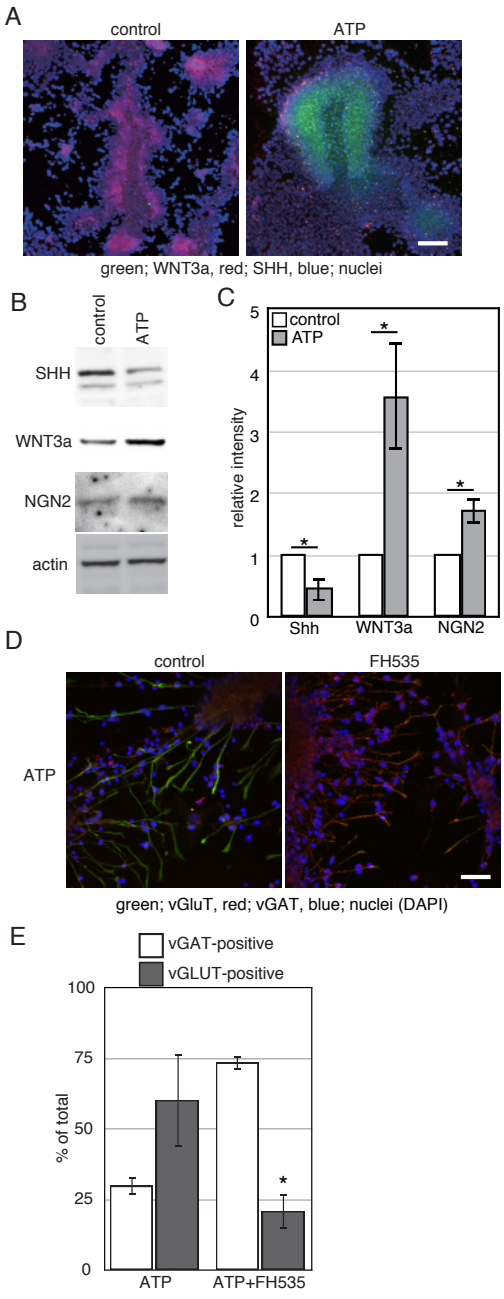

Figure S1. WNT3a expression in differentiating ES cells after nucleotide treatment, related to Figure 1.

The mouse ES cell line CGR8 was cultured in a dish coated with gelatine and differentiation was induced by depletion of growth factors. (**A**) On Day 5 of differentiation, cells were fixed and expression of the indicated proteins was examined. Differentiating mouse ES cells formed a neural tube-like structure, and expression of Shh was detected. However, when ATP was added, cells

forming the neural tube-like structure expressed WNT3a. Bar indicates 50 μm. (**B**) Cells were harvested on Day 5 of differentiation, and cell extracts were prepared. Twenty μg of protein was analysed by SDS-PAGE and expression of the indicated proteins was examined. (**C**) The intensities of signals in (B) were assessed with Image J ver. 1.45n. The graph shows the mean of three independent experiments and the error bars indicate standard error. The intensities of bands in control cells were set at 1. Asterisk indicates $p<0.05$ (student t-test). The expression level of SHH was decreased approximately half by the ATP treatment. Reciprocally, the expression of WNT3a was increased 3 times. Expression of a transcription factor neurogenin 2 (NGN2) was also increased after 5 days of ATP treatment. NGN2 is a member of the cascade of transcription factors directing glutamatergic subtype selection of newborn neurons (Berninger et al., 2007; Heinrich et al., 2010; Roybon et al., 2010). (**D**) Differentiation of mouse ES cells was induced for 14 days on gelatine in the presence of 20 μM of the indicated nucleotides, and with or without 10 μM of an WNT/beta-catenin inhibitor FH535. Bar indicates 50 μm. (**E**) Cells positive for vGAT or vGluT in (D) were quantified as described in the Materials and Methods. The graph shows the mean of three independent experiments and the error bars indicate standard error. Asterisk indicates $p<0.05$, relative to "ATP" (student t-test). Treatment with an inhibitor of the canonical WNT signalling FH535 blocked the nucleotide-induced vGluT expression. These results indicate that WNT signalling appears to be involved in the pathway of nucleotide-dependent vGluT expression.

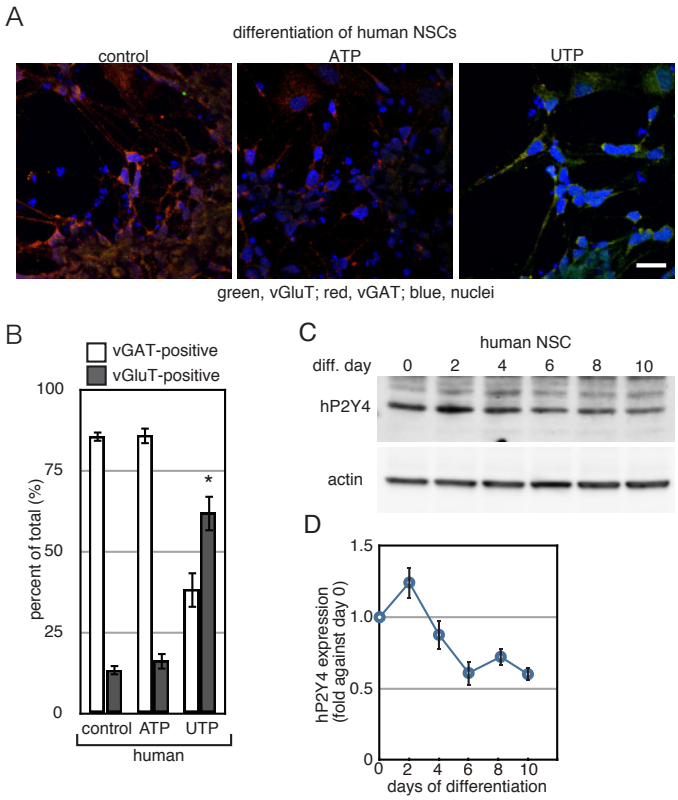

Figure S2. Effects of nucleotides on the differentiation of human NSCs, related to Figure 2 and 4.

(**A**) Differentiation of NSCs derived from human ES cells was induced for 10 days in the presence of 20 μM of the indicated nucleotides. Bar indicates 25 μm. (**B**) Cells positive for vGAT or vGluT in (A) were quantified as described in the Experimental Procedures section. The graph shows the mean of three independent experiments and the error bars indicate standard error. Asterisk indicates $p<0.05$, relative to control (student t-test). UTP, but not ATP, induced vGluT expression in cells differentiated from human NSCs, while both UTP and ATP induced vGluT expression in neurons differentiated from mouse ES cells and NSCs (Figure2A). UTP, but not ATP, can activate human P2Y4, whereas both UTP and ATP can activate mouse P2Y4 (Communi et al., 1995; Nguyen et al., 1995; von Kugelgen, 2006). Human P2Y2, as well as mouse P2Y2, can be activated with both ATP and UTP. (**C**) Cell extracts were prepared from cells on the indicated differentiation days, and samples (20 μg of proteins) were analysed by SDS-PAGE. (**D**) Intensities of signals in (C) were assessed with Image J ver. 1.45n. The graph shows the mean of three independent experiments and the error bars indicate standard error. Expression of P2Y4 was increased on the

day 2 of differentiation, and then decreased gradually in the neuronal differentiation (Supplemental Figure 2C and D). These results suggest that P2Y4-mediated induction of vGluT is conserved between mouse and human.

Supplemental Experimental Procedures

Antibodies

Anti-NGN2 antibody (AB3314) was purchased from Millipore. Anti-WNT3a antibody (2721S) was purchased from Cell Signaling. Anti-SHH antibody (MAB464) was purchased from R&D. Anti-human P2Y4 (BS2953) was purchased from Bioworld. Anti-human vGluT (MAB5686) was purchased from Abnova.

Human NSC culture and differentiation induction

Human NSCs derived from the H9 human ES cell line were purchased from Invitrogen. Human NSCs were cultured on dishes coated with polyornitine and laminin, and their differentiation was induced by depletion of FGF2 and EGF according to the manufacturer's recommendation.

Supplemental references

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