Reactive Oxygen Species Mediate the Potentiating Effects of ATP on GABAergic Synaptic Transmission in the Immature Hippocampus*

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Reactive oxygen species (ROS) constitute important signaling molecules in the central nervous system. They regulate a number of different functions both under physiological conditions and under pathological conditions. Here we tested the hypothesis that in the immature hippocampus ATP, the most diffuse neurotransmitter in the brain, modulates synaptic transmission via ROS. We show that ATP, acting on metabotropic P2Y1 receptors, increased the frequency of GABA_A-mediated spontaneous postsynaptic currents (SPSCs) in CA3 principal cells, an effect that was prevented by the antioxidant N-acetyl-cysteine or by catalase, an enzyme that breaks down H_2O_2. The effect of ATP on SPSCs was mimicked by H_2O_2 or by the pro-oxidant, via ROS. We show that ATP, acting on metabotropic P2Y1 neurotransmitter in the brain, modulates synaptic transmission.

Growing evidence suggests that, in physiological conditions, ROS regulate neuronal signaling both in the central and in the peripheral nervous system. Although at the periphery, ROS contribute to the inhibitory effect of ATP on quantal acetylcholine release from motor nerve endings, in the central nervous system, ROS produce both enhancement and depression of synaptic transmission. In pathological conditions, increased amounts of ROS are involved in apoptosis and neurodegenerative disorders such as Parkinson and Alzheimer disease.

In the nervous system, ROS are produced mainly by microglia and by astrocytes. In cultured astrocytes, activation of metabotropic P2Y1 receptors by extracellular ATP induces ROS via membrane-bound NADPH oxidase. However, it is unclear whether a similar mechanism also occurs in situ and whether ATP-induced ROS production in astrocytes can reach target neurons and affect synaptic transmission.

In the present study, electrophysiological and imaging techniques were combined to investigate ROS effects on GABAergic transmission in principal cells on acute hippocampal slices from newborn rats. Early in postnatal life, spontaneous activity in the hippocampus is characterized by network-driven membrane potential oscillations, the so-called giant depolarizing potentials (GDPs)), and spontaneous ongoing postsynaptic potentials. Both types of activity are required for proper wiring of developing circuits. During this period, ROS signaling should be more pronounced because of the relative deficiency of superoxide dismutase and glutathione peroxidase, known as powerful scavengers of ROS. In our previous studies, we have demonstrated that GDPs and GABA_A-mediated spontaneous postsynaptic potentials are modulated by ATP, which is endogenously released during neuronal activity. Since ATP may induce ROS production in glial cells, it is important to test whether ROS induction is involved in the action of ATP. We found that ATP, via metabotropic P2Y1 receptor, excites astrocytes and induces the release of diffusible ROS, which in turn facilitate GABA release onto CA3 pyramidal cells in the immature hippocampus.

EXPERIMENTAL PROCEDURES

Slice Preparation—Experiments were performed on hippocampal slices obtained from postnatal day 1 (P1)–P6 Wistar rats as described. Briefly, animals were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 g/kg). All experiments were carried out in accordance with the institutional guidelines for the care of animals.
with the European Community Council Directive of November 24, 1986 (86/609/EEC) and were approved by local authority veterinary service. The brain was removed from the skull and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing 130 mM NaCl, 3.5 mM KCl, 1.2 mM NaH2PO4, 25 mM NaHCO3, 1.3 mM MgCl2, 2 mM CaCl2, and 25 mM glucose and then saturated with 95% O2 and 5% CO2 (pH 7.3–7.4). Transverse hippocampal slices (500 μm thick) were cut with a vibratome and stored at room temperature in a holding bath containing the same solution as above. After a recovery period of at least 1 h, an individual slice was transferred to the recording chamber, where it was continuously superfused with oxygenated ACSF at a rate of 2–3 ml/min at 33–34 °C.

**Electrophysiological Recordings**—Electrophysiological experiments were performed from CA3 pyramidal cells using the whole-cell configuration of the patch clamp technique in voltage clamp mode. Patch electrodes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, Germany). They had a resistance of 5–7 megohms when filled with an intracellular solution containing (in mM): 140 KCl, 1 MgCl2, 1 NaCl, 1 EGTA, 2 HEPES, and 2 K2ATP. The pH was adjusted to 7.3 with KOH. Recordings were made with a patch clamp amplifier (Axopatch 1D; Axon Instruments). The whole-cell capacitance was fully compensated, and the series resistance (10–20 megohms) was compensated at 75–80%. The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiment. Substances and drugs used were ATP and DPCPX (purchased from Tocris Cookson; Bristol, UK) and N-acetyl-1-cysteine (NAC), iron sulfate heptahydrate, 6,7-dinitroquinoxaline-2,3-dione (DNQX), catalase, and H2O2 (purchased from Sigma, Milan, Italy). Solutions of NAC and Fe2+1 were prepared immediately before use. All drugs were dissolved in artificial cerebrospinal fluid; DNQX was dissolved in MeSO. The final concentration of MeSO in the bathing solution was 0.1%. At this concentration, MeSO alone did not modify the shape or the kinetics of synaptic currents. Drugs were applied in the bath via a three-way tap system, by changing the superfusion solution to one differing only in its content of drug(s). The ratio of flow rate to bath volume ensured complete exchange within 1.5–2 min.

**Analysis of Electrophysiological Data**—Data were stored on magnetic tape and transferred to a computer after digitization with an A/D converter (Digidata 1200, Axon Instruments, Foster City, CA). Data were sampled at 20 kHz and filtered with a cut-off frequency of 1 kHz. Acquisition and analysis were performed with Clampex 7 (Axon Instruments, Foster City, CA). The analysis of SPSCs was performed with the Mini Analysis Program (Synaptosoft Inc., Decatur, GA). General principles and details on the analysis of synaptic currents can be found elsewhere (18). The analysis of spontaneous synaptic currents was performed on sequences with at least 100 events each. Signals were repeatedly averaged for at least 15 min before infusion of agonists or antagonists to ensure stable baseline conditions. Spontaneous events were detected using amplitude thresholds set as a multiple (4–5 times) of the standard deviation of the noise. The cumulative amplitude and interevent plots obtained for cells in control and after drug application were compared using the Kolmogorov-Smirnov test. Values are given as mean ± S.E. Significance of differences was assessed by Student’s t test or Mann-Whitney test. The significance level is p < 0.05.

**Imaging of Calcium and ROS**—Hippocampal slices were incubated for 1 h in continuously oxygenated ACSF containing 30 μM of the cell-permeable H2O2-selective fluorescent dye carboxy-2,7-dichlorodihydrofluorescein diacetate (DCF, Molecular probes, Eugene, OR). After cleavage by cell esterases and oxidation, this probe was monitored using excitation sources and filters appropriate for fluorescein. Cells were imaged with the Bio-Rad Radiance 2100 laser scanning confocal microscope using the 488-nm laser line. Emitted green fluorescence was collected through a 520 long-pass filter. Bio-Rad software was used for image acquisition. Some of the major artifacts when DCF is used are photo-oxidation and photo-damage (19). To prevent these artifacts (see also Ref. 20), we used multiphoton excitation of DCF with Mai-Tai pulsed infrared laser (Spectra Physics). Excitation wavelength was set to 800 nm, and emission was collected through the same long-pass filter as in the single-photon confocal imaging mode. Another artifact that is not often taken into account in long-lasting experiments is leakage of the dye from cells (21). Therefore, we kept the dye constantly present in bath solution at the same concentration as that used during incubation. Prior to Ca2+ imaging experiments, cells were incubated for 1 h in oxygenated ACSF containing 4 μM fluo-4-AM (Molecular Probes). An additional period of 30 min was allowed for de-esterification of the dye. Under these incubation conditions, astrocytes were loaded preferentially, whereas the dye uptake by neurons was minimal (22, 23). Images were acquired every 5 s using the same filters as indicated above. Fluorescence intensity in background-subtracted frame averages was normalized and presented as (ΔF/F) = [(intensity-basal)/(basal)]*100%.

**RESULTS**

The Antioxidant NAC Prevents the Facilitatory Action of ATP on GABAergic Transmission—During the first postnatal week, whole-cell recordings from CA3 pyramidal neurons revealed two types of spontaneous activity: GDPs and SPSCs. Although GDPs are network-driven events generated by the synergistic action of glutamate and GABA (13), SPSCs are almost exclusively postsynaptic currents (SPSCs) from 4.8 to 1.0 μA (n = 6, p < 0.01). ATP application did not affect the amplitude of SPSCs (the peak amplitude was 30.8 ± 8.6 μA before and during ATP application, respectively; n = 6, p > 0.05; Fig. 1F). Moreover, the potentiation effect of ATP was prevented by the P2Y2-selective antagonist MRS-2179 (10 μM, see Fig. 4 of Ref. 14), indicating that it was mediated by metabotropic P2Y1 receptors.

To test whether the potentiating effect of ATP on GABAergic SPSCs was mediated by ROS, we used the antioxidant NAC,
which is a widely used ROS scavenger (5, 27). The action of ATP on SPSCs was prevented by NAC (Fig. 1, C, D, and E). In the presence of NAC (100 μM), ATP failed to increase the frequency of SPSCs (the frequency was 5.1 ± 1.1 and 5.9 ± 1 Hz before and during ATP, respectively, p > 0.05, n = 6). NAC per se did not significantly modify the frequency (this was 5.8 ± 1.2 and 5.1 ± 1.1 Hz, before and during NAC, respectively, n = 6; p > 0.05) or the amplitude of SPSCs (this was 16.7 ± 3.6 and 14.8 ± 3.2 pA before and during superfusion of NAC, respectively; p > 0.05). Similar results were obtained with NAC (1 mM, data not shown).

Consistent with the data obtained with NAC, catalase, an enzyme known to specifically break down H₂O₂ (28, 29), prevented the potentiating action of ATP on SPSCs (Fig. 2). Thus, in the presence of catalase (1200 units/ml), the frequency or amplitude of SPSCs was not modified by ATP (50 μM); the frequency of SPSCs was 6.3 ± 1.3 and 7.5 ± 1.7 Hz in the absence or presence of ATP, respectively; n = 7; p > 0.05, whereas the amplitude was 21.7 ± 4.8 and 26.2 ± 6.1 pA in the absence or presence of ATP, respectively; n = 7; p > 0.5). Altogether, these results suggest that the action of ATP on spontaneous GABAergic synaptic currents is mediated by reactive oxygen species.

H₂O₂ Mimics the Potentiating Effects of ATP on Spontaneous GABAergic Events—To further explore the involvement of ROS in ATP action, we tested the effect of exogenous H₂O₂, a cell-permeant form of ROS, on SPSCs. Similarly to ATP, a low concentration of H₂O₂ (30 μM) enhanced the frequency of SPSCs from 4.0 ± 0.6 to 5.8 ± 0.8 Hz (n = 5, p < 0.05; Fig. 3, A and C) without significantly altering their amplitude (on average, the peak amplitude of SPSCs was 39.6 ± 8.3 and 44.8 ± 11.8 pA

![FIGURE 1. NAC prevents ATP-induced facilitation of GABAergic SPSCs.](image1)

![FIGURE 2. Catalase prevents the potentiating effect of ATP on SPSCs.](image2)
before and during H$_2$O$_2$, respectively ($p > 0.05$; Fig. 3D). The effects of H$_2$O$_2$ on SPSCs were prevented by NAC (1 mM); the frequency of SPSCs was 4.2 ± 0.6 and 4.6 ± 0.6 Hz in NAC and in the presence of NAC plus H$_2$O$_2$, respectively; $n = 6$; $p > 0.05$; Fig. 3, B and C), indicating that they were mediated via ROS (31).

Endogenously Released ATP Affects Synaptic Release of GABA from GABAergic Interneurons—It is known that, in the presence of Fe$^{2+}$, H$_2$O$_2$ is readily converted via the Fenton reaction into the hydroxyl radical (OH$^-$), which is the strongest oxidant among other ROS (31). In this set of experiments, we tested whether Fe$^{2+}$ can enhance synaptic activity via endogenous ROS. Fe$^{2+}$ in a relatively low concentration (10 μM) increased the frequency of SPSCs from 6.0 ± 0.7 to 8.7 ± 1.0 Hz ($n = 7$; $p < 0.05$; Fig. 4, B and C). Iron did not change the amplitude of SPSCs (the peak amplitude of synaptic currents was 35.8 ± 9.5 and 32.9 ± 9.5 pA before and during application of Fe$^{2+}$, respectively; $p > 0.05$ (Fig. 4C)). The potentiating effect of iron on SPSCs was prevented by NAC (1 mM), indicating that it was mediated by ROS (Fig. 4, A and C). Thus, the frequency of SPSCs was 7.9 ± 0.9 and 8.6 ± 1.0 Hz in the presence of NAC and NAC plus iron, respectively ($n = 6$; $p > 0.05$), whereas the amplitude was 13.4 ± 3.6 and 13.8 ± 3.8 in the presence of NAC and NAC plus iron, respectively ($n = 6$; $p > 0.05$).

![Figure 3](image-url)  
**FIGURE 3.** H$_2$O$_2$ mimics the potentiating effect of ATP on GABAergic SPSCs. A, representative traces (obtained at P6) of spontaneous GABA$_A$-mediated synaptic currents recorded before and during application of H$_2$O$_2$ (30 μM). B, in another neuron (at P5), NAC (1 mM) prevented the action of H$_2$O$_2$. C and D, mean frequency (C) and amplitude (D) values of SPSCs (normalized to controls, dashed line) obtained in the presence of H$_2$O$_2$ and NAC plus H$_2$O$_2$ ($n = 6$). *, $p < 0.05$ (Student's t test).

![Figure 4](image-url)  
**FIGURE 4.** Endogenously released ATP enhances the frequency of SPSCs via ROS production. A, Fe$^{2+}$ did not affect the frequency or amplitude of SPSCs when applied in the presence of NAC. Traces were obtained (at P5) in control, in the presence of NAC (1 mM), and in the presence of NAC plus Fe$^{2+}$ (10 μM). B, representative traces of GABA$_A$-mediated SPSCs recorded (at P6) in control conditions and during bath application of Fe$^{2+}$ (10 μM) and Fe$^{2+}$ plus MRS-2179 (10 μM). C, mean frequency (white columns) and amplitude (black columns) values of SPSCs obtained in the presence of Fe$^{2+}$ ($n = 7$, left), NAC plus Fe$^{2+}$ ($n = 5$, middle) and Fe$^{2+}$ plus MRS-2179 (MRS) ($n = 4$, right). Data were normalized to pre-iron, NAC, and iron values respectively (dashed lines). *, $p < 0.05$ (Student's t test and Kolmogorov-Smirnov test).

To test whether Fe$^{2+}$-induced enhancement of synaptic activity was mediated by endogenously released ATP, we tried to antagonize the effect of Fe$^{2+}$ with the selective P2Y$_1$ receptor blocker MRS-2179. Indeed, MRS-2179 at the concentration of 10 μM, which per se did not significantly modify the frequency of spontaneous synaptic events (14), strongly antagonized the facilitatory action of Fe$^{2+}$ on spontaneous GABA$_A$-mediated synaptic currents (Fig. 4, B and C). The frequency of SPSCs was 9.5 ± 0.8 and 6.9 ± 0.9 Hz in the presence of Fe$^{2+}$ and Fe$^{2+}$ plus MRS-2179, respectively, whereas the amplitude was 45.5 ± 16.2 and 39.5 ± 11.1 pA in the presence of Fe$^{2+}$ and Fe$^{2+}$ plus MRS-2179, respectively. These data indicate that endogenous ATP acting on P2Y$_1$ receptors is involved in iron-induced modulation of synaptic transmission.

Ca$^{2+}$ Imaging in Astrocytes and the Action of NAC—The increase in intracellular calcium in astrocytes has been shown to be an important step in the generation of ROS via NADPH oxidase (10). Previous experiments from acute hippocampal slices obtained from adult rats have revealed calcium transients in astrocytes following activation of P2Y$_1$ receptors (25, 32). To test whether, in immature hippocam-
ROS Control GABA Release

FIGURE 5. ATP-induced Ca^{2+} transients in CA3 astrocytes. A–C, fluorescence changes in fluo-4AM loaded cells in response to 100 μM ATP application (bars). ATP-induced Ca^{2+} response was blocked by the P2Y_1 receptor antagonist MRS-2179 (50 μM, A) but persisted in the presence of NAC (1 mM, B) or catalase (2000 units/ml, C). D, quantification of the data illustrated in A–C. ATP-induced Ca^{2+} transients were normalized to those observed in control conditions (n = 14) prior to the addition of either MRS-2179 (n = 9; p < 0.001), NAC (n = 14; p = 0.15) or catalase (n = 14). ***, p < 0.001.

FIGURE 6. Astrocytes generate ROS in response to ATP application. A, confocal image of DCF-loaded cells in CA3 region of a P3 hippocampal slice. Fluorescence increase upon 100 μM ATP application (right panel) versus a pre-application time point (left panel) was more pronounced in cell 2, which was consequently identified as astrocyte by patch clamping. B, whole-cell current clamp recordings from the two cells shown in A. Note that in cell 1, spikes were generated in response to current injection (shown by the current step diagram), whereas much stronger current injections failed to induce action potentials in cell 2, which was consequently identified as astrocyte by patch clamping. C, time course of changes in DCF fluorescence under multiphoton excitation in response to application of ATP (100 μM, bar) in cells identified as astrocytes (filled circles) and neurons (open triangles). Note that multiphoton excitation minimizes photo-oxidation and photo-bleaching of DCF, thus revealing the rapid onset of the DCF signal and its fast recovery to baseline upon ATP washout. D, quantification of the increase in fluorescence intensity in response to ATP (n = 8) or to H_2O_2 (n = 40). Both ATP and H_2O_2 induced significant increase in DCF fluorescence (p = 0.003 and p < 0.001 respectively).

DISCUSSION

The main finding of the present study is that in the immature hippocampus, ATP, a widely distributed transmitter and neuromodulator, controls GABA release onto CA3 principal cells via induction of ROS. Imaging experiments with the selective ROS-sensitive dye DCF have clearly demonstrated that ROS are produced by glial cells following activation of P2Y_1 receptors by
ATP. Thus, purinergic modulation of synaptic transmission in the brain involves cross-talk between glia and neurons mediated by ROS acting as diffusible messengers.

**ROS Are Involved in ATP-induced Modulation of GABAergic Transmission**—During the first postnatal week, when associational commissural fibers are still poorly developed (34, 35), CA3 pyramidal cells receive a strong GABAergic innervation from the mossy fibers and GABAergic interneurons (36). Therefore, during this period, spontaneous synaptic activity recorded from CA3 principal cells is mediated mainly by GABA acting on GABA_A receptors, as demonstrated by the observation that in the presence of GABA_A receptor antagonists, spontaneous glutamatergic events are merely detectable (24). In previous work, we have demonstrated that either endogenously released or exogenously applied ATP, via the activation of metabotropic P2Y_1 receptors, exerts a powerful excitatory action on spontaneous GABA_A-mediated synaptic events (14). The present data provide evidence that ATP-induced increase in GABA release is mediated by ROS because: (i) the effect of ATP was prevented by NAC, a precursor of the most efficient endogenous antioxidant glutathione (27); (ii) the potentiating effect of ATP was abolished by catalase, an enzyme that specifically breaks down H_2O_2 (28, 29); (iii) the effect of ATP on GABA release was mimicked by low concentrations of H_2O_2, a mild oxidant that, unlike other ROS, is relatively stable and capable of diffusing at relatively long distances (4); and (iv) a similar facilitatory effect was produced by the pro-oxidant Fe^{2+}, which, via the Fenton reaction, catalyzes the transformation of H_2O_2 into highly reactive hydroxyl radicals (31).

In addition, imaging experiments with a selective ROS-sensitive dye have provided direct evidence that ROS production is triggered by ATP via activation of P2Y_1 receptors. However, the present experiments do not allow excluding the possibility that other radicals are involved in ATP action on synaptic transmission. It has been recently demonstrated that ATP can induce in astrocytes the production of nitric oxide (37), which may interact with ROS (38). Thus, nitric oxide, either alone or in cooperation with superoxide (to produce highly aggressive ONOO^- (28, 39)), can control GABAergic signaling in the hippocampus. Altogether, our data reveal that, in the immature hippocampus, ATP controls the local network through ROS production.

**Endogenous ATP Contributes to ROS Generation**—ATP is the most ubiquitous molecule stored at high concentrations (mM range) in synaptic vesicles from which it can be released in an activity-dependent manner (40–42). We have previously shown that, in the immature hippocampus, synchronized network activity such as GDPS enhances the level of endogenous ATP, which in turn regulates GDP induction (14). Endogenous ATP can also be released in a Ca^{2+}-dependent way from astrocytes (Fig. 7), which abundantly express purinergic receptors (25, 32, 43, 44). Accumulation of endogenous ATP in the extracellular space may lead to ROS production by astrocytes and facilitation of GABA release from interneurons. Consistent with this view, Fe^{2+}-induced facilitation of synaptic transmission was prevented when the action of endogenous ATP on P2Y_1 receptors was reduced by MRS-2371, a specific P2Y_1 antagonist (Fig. 7). It should be mentioned that P2Y_1 receptor can also be activated by ADP, the breakdown product of ATP (25, 32, 45, 46). Therefore, we cannot exclude the possibility that endogenous ADP acting on P2Y_1 receptors contributes to ROS generation by astrocytes. In turn, the contribution of ADP would be dependent on the activity of ectoATPases, which control the rate of ATP breakdown (14, 45).

Our experiments also reveal facilitation of GABA release by endogenous H_2O_2, which is more evident in the presence of Fe^{2+}. It is worth noting that in newborns, there is an excess of free iron (16), which is a cofactor for the generation of highly reactive hydroxyl radicals.

**Sources of ROS in the Hippocampus**—What are the cellular sources of ROS? Possible candidates are astrocytes and interneurons since both of these cells express metabotropic P2Y_1 receptors (25, 32). However, the present work based on DCF imaging from brain slices combined with electrophysiological recordings has clearly identified astrocytes as the main cellular source of ROS. In the present study, we did not analyze the molecular mechanism of ROS generation. Nevertheless, recent studies on cultured astrocytes have shown that ATP-induced ROS are generated mainly by membrane NADPH oxidase (10).

**Physiological Implication of ROS in the Immature Hippocampus**—The present data demonstrate that the hippocampus of newborn rats is very sensitive to ROS since H_2O_2 at low concentrations was effective in enhancing the frequency of spontaneous GABA_A-mediated synaptic events. It is known that in particular situations such as reperfusion after ischemia, endogenous H_2O_2 can reach in certain brain regions a local concentration up to 150 mM (47). Such high concentrations of H_2O_2 may have a variety of adverse effects or even kill the neurons (48). The high sensitivity of newborns to ROS may result not only from the relative deficiency of superoxide dismutase and glutathione peroxidase in the immature brain, known as
the main scavengers of ROS, but also from the excess of free iron (16). Consistent with this, in the present work, even low micromolar concentrations of Fe²⁺ were able to modulate the action of endogenous ATP, promoting facilitation of GABA release into pyramidal cells. As already mentioned, at early developmental stages, GABAergic transmission provides the major excitatory drive to pyramidal cells (14, 36, 49). Fig. 7 shows that ATP released from astrocytes induces peroxide via activation of P2Y₁ receptors coupled to intracellular calcium rise. In the presence of Fe²⁺, H₂O₂ is converted in hydroxyl radicals, which in turn facilitate GABA release from mossy fibers and GABAergic interneurons onto principal cells. It is worth noting that ROS act mainly presynaptically without modifying postsynaptic responsiveness. At the neumromuscular junction, ROS were found to affect acetylcholine release, probably interfering with the vesicle fusion protein SNAP25 (50). However, this protein is not expressed in hippocampal GABAergic synapses (51), suggesting another target for modulation of GABAergic synaptic currents by ROS. Considering that a single astrocyte may control ~140,000 synapses (33) and that neighboring astrocytes communicate by means of intercellular Ca²⁺ waves (43), the release of ROS from astrocytes may result in a widespread and synchronized synaptic modulation.

In the immature hippocampus, synaptic GABAergic activity is required for proper wiring of developing circuits (15). This implies that any change in pro-oxidant levels may interfere with ATP-induced ROS production and synaptic transmission, thus contributing to information processing at the network level.

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