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Thrombin induces ischemic LTP (iLTP): implications for synaptic plasticity in the acute phase of ischemic stroke

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Acute brain ischemia modifies synaptic plasticity by inducing i*schemic long-term potentiation* (iLTP) of synaptic transmission through the activation of N-Methyl-D-aspartate receptors (NMDAR). Thrombin, a blood coagulation factor, affects synaptic plasticity in an NMDAR dependent manner. Since its activity and concentration is increased in brain tissue upon acute stroke, we sought to clarify whether thrombin could mediate iLTP through the activation of its receptor Protease-Activated receptor 1 (PAR1). Extracellular recordings were obtained in CA1 region of hippocampal slices from C57BL/6 mice. *In vitro* ischemia was induced by acute (3 minutes) oxygen and glucose deprivation (OGD). A specific *ex vivo* enzymatic assay was employed to assess thrombin activity in hippocampal slices, while OGD-induced changes in prothrombin mRNA levels were assessed by (RT)qPCR. Upon OGD, thrombin activity increased in hippocampal slices. A robust potentiation of excitatory synaptic strength was detected, which occluded the ability to induce further LTP. Inhibition of either thrombin or its receptor PAR1 blocked iLTP and restored the physiological, stimulus induced LTP. Our study provides important insights on the early changes occurring at excitatory synapses after ischemia and indicates the thrombin/PAR1 pathway as a novel target for developing therapeutic strategies to restore synaptic function in the acute phase of ischemic stroke.

Cerebral ischemic events affect brain physiology at a diverse range of time scales[1]. Several investigations throughout recent years have identified signaling pathways triggered by ischemic stroke, which ultimately lead to excitotoxicity and cell death[2]. However, the cellular and molecular mechanisms of the early, yet likely reversible, post ischemic changes remain incompletely understood[3]. The short time scale at which these events occur in the brain is a major obstacle for their investigation using animal models of disease. Contrarily, oxygen-glucose deprivation (OGD) of brain slices *in vitro* has been proposed to be a valid alternative to address changes in neuronal physiology in the early post ischemic period[4–6]. Using this experimental approach, it has been demonstrated that a brief exposure (i.e., a few minutes) to OGD potentiates synaptic transmission at hippocampal CA3-CA1 synapses (iLTP) without causing neuronal cell death[4,7,8]. iLTP is a pathological form of synaptic plasticity, which depends on NMDAR activation and involves both presynaptic and postsynaptic loci[4]. Therefore, it has been proposed that iLTP could have a major impact on the functional reorganization of neuronal networks during the early phase of ischemic stroke.

During acute ischemia, thrombin concentrations and activity rise in the ischemic core leading to cognitive deficits[9,10]. Thrombin, a serine protease playing an essential role in the blood coagulation cascade, has been implicated in the regulation of synaptic plasticity in the brain[11–15]. Specifically, exposure of hippocampal slices to thrombin induces a slow onset LTP through activation of its receptor Protease-Activated receptor 1 (PAR1) and consequent potentiation of NMDAR function[12,16,17]. Thrombin mediated slow onset LTP affects the ability of neurons to express further synaptic plasticity. While it is conceivable that thrombin induced LTP may occur in a pathological setting due to the elevated thrombin concentrations in the brain[17,18], it has not yet been addressed whether acute brain ischemia could affect synaptic plasticity via a thrombin mediated signaling pathway.

In the present study, we demonstrate that OGD exposure of hippocampal slices is accompanied by an increase in thrombin activity, which triggers iLTP and impairs the ability of neurons to express further LTP. Since iLTP is blocked when thrombin activity or PAR1 signaling is inhibited we conclude that thrombin causes iLTP following acute ischemia and hypothesize that counteracting thrombin signaling in the brain during the early phase of stroke may improve synaptic plasticity. Hence, thrombin- and/or PAR1-inhibition may support the functional reorganization of neuronal networks during the early phase after stroke.

Methods

All the described methods were carried out in accordance with the approved guidelines. *Animals*: Animal handling as well as all described experiments were performed in accordance and approved by the Institutional Animal Care and Use Committee of The Chaim Sheba Medical Center (Tel HaShomer, Israel), which adheres to the Israeli law on the use of laboratory animals and NIH rules. Four/five-months old male C57BL/6 mice were used for the experiments. In some experiments, age-matched male PAR1-KO mice (provided by Prof. Yair Reisner, Weizmann Institute of Science, Israel) and their wildtype littermates were used. *Electrophysiology*: Extracellular recordings in hippocampal slices were performed as previously reported[19,20]. Briefly, following anesthesia with ketamine/xylazine (dosage of 100 and 10 mg/Kg, respectively), animals were rapidly decapitated, the brain removed and 400 μm slices prepared using a vibroslicer. Slices were incubated for 1.5 h in a humidified, carbogenated (5% CO2 and 95%O2) gas atmosphere at $33 \pm 1^{\circ}C$ and perfused with ACSF [containing (in mM)124 NaCl, 2 KCl, 26 $NaHCO_3$, 1.24 KH_2PO_4, 2.5 $CaCl_2$, 2 $MgSO_4$, and 10 glucose, pH 7.4] in a standard interface chamber. Recordings were made with a glass pipette containing 0.75MNaCl (4 MΩ) placed in the stratum radiatum CA1. Stimulation was evoked using a pulse stimulator and delivered through a bipolar nichrome electrode. In a set of experiments, two sets of bipolar nichrome electrodes placed on either side of the recording electrode were used in order to stimulate two independent channels in a single slice[28]. LTP was induced by high-frequency stimulation (HFS) consisting of 100 pulses at twice the test intensity, delivered at a frequency of 100 Hz (100 Hz, 1 s). *In vitro* ischemia was induced by switching to an artificial cerebrospinal fluid solution in which sucrose replaced glucose, gassed with 95% N_2 and 5% CO_2[21]. Before applying the tetanic stimulation, baseline values were recorded at a frequency of 0.033 Hz. Responses were digitized at 5 kHz and stored on a computer. Offline analysis and data acquisition was performed by Spike2 software. Excitatory Post Synaptic Potential (EPSP) slope changes after tetanic stimulation were calculated with respect to baseline. There were no systematic differences in the magnitudes of the baseline responses in the different conditions. *Thrombin activity assay*: Thrombin activity was assessed as previously described[9,22,23]. Slices were frozen in liquid nitrogen and stored at $-80^{\circ}C$ until used for thrombin activity evaluation. Thrombin activity was measured by a fluorimetric assay quantifying the cleavage of the synthetic peptide substrate Boc-Asp(OBzl)-Pro-Arg-AMC (I-1560, Bachem, Switzerland, 13 μM final concentration). Measurements were performed by the Infinite 2000 microplate reader (Tecan, infinite 200, Switzerland) with excitation and emission filters of 360 ± 35 and 460 ± 35 nm, respectively. *Western blot detection*: Hippocampal slices were homogenized in RIPA buffer [containing in mM: 50 TRIS HCl pH8, 150 NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS] using pestle motor mixer. The samples were centrifuged (14,000 g, 10 minutes, $4^{\circ}C$) and the supernatants were separated. 8 μg from each sample were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes. Membranes were incubated with goat anti thrombin antibody (diluted 1:200, Santa Cruz Biotechnology, sc-23335)[10] over night at $4^{\circ}C$ and washed. Membranes were then incubated at room temperature with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories). Protein bands were detected by a peroxidase-based ECL method. Following the detection the membranes were stripped and re-incubated with mouse anti-actin antibody (1:10,000, 69100 MP) and re-detected by ECL. Analysis of the protein bands density was performed with ImageJ software. *Quantitative Real Time (RT) PCR*: Total RNA was extracted using Biorad Aurum 732-6820 (Bio-Rad, CA, USA). Eight hundred nanograms of total RNA were used for reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rhenium, Israel). qRT-PCR was performed on the 7900HT Fast Real Time PCR System (Applied Biosystems, Rhenium, Israel), Prothrombin (PT) mRNA levels were examined using FastStart Universal SYBR Green Master (ROX) (Roche, Mannheim, Germany), with the following primer sequences: PT: 5' CCGAAAGGGCAACCTAGAGC, 5' GGCCCAGAACACGTCTGTG. Hypoxanthine Guanine Phosphoribosyltransferase (HPRT; 5' TGAAAGACTTGCTCGAGATGTCA, 5' CACACAGAGGGCCACAATGT) served as a reference gene in this analysis (Sigma-Alrich, Rehovot, Israel). A standard amplification program was used (1 cycle of $50^{\circ}C$ for 2 min, 1 cycle of $95^{\circ}C$ for 10 minutes, 40 cycles of $95^{\circ}C$ for 15 s and $62^{\circ}C$ for 60 s). *Statistics*: All numerical data are expressed as mean ± SEM, unless otherwise indicated. Statistical analysis was performed by applying a Student's *t* test for paired or unpaired data, as the case may be (Origin 8.0). *p* values of <0.05 were considered a significant difference between means.

Results

Oxygen-glucose deprivation induces NMDAR dependent ischemic LTP (iLTP) and increases thrombin activity in acute hippocampal slices. A brief (3 minutes) episode of *in vitro* OGD caused a transient reduction in EPSP slopes recorded in stratum radiatum of hippocampal area CA1 (Fig.1a). This was followed by a long-term increase of EPSP slope (up to 1.62 ± 0.078 at 10 minutes after recovery, n = 12 slices; Fig. 1A), reflecting ischemic LTP (iLTP) of excitatory synaptic transmission[5,24]. This form of synaptic plasticity requires NMDAR activation[21], and hence was blocked in presence of the NMDAR antagonist APV (50 μM; 1.05 ± 0.084 at 10 minutes after recovery, n = 12; $p < 0.001$; Fig. 1b).

To test for the effects of transient OGD on thrombin in our hippocampal slices, which are blood-free preparations, thrombin activity was measured using a specific enzymatic assay, as previously shown[9,22,23]. Strikingly, a ~4fold increase in thrombin activity was measured at 10 minutes following OGD recovery compared to control slices, not exposed to OGD (control: 474.15 ± 166.43; OGD: 2054.82 ± 574.34; n = 12; $p < 0.01$; Fig. 1c). Specificity of these experiments was confirmed using the selective thrombin inhibitor α-NAPAP (1 μM). Indeed, in presence of α-NAPAP thrombin activity measured at 10 minutes following OGD was not significantly increased (control: $7.05E^{-04} \pm 1.42E^{-08}$; OGD: $6.7E^{-04} \pm 2.68E^{-08}$; n = 12; Fig.1c), thus indicating that OGD specifically increased thrombin activity in hippocampal slices. In a different set of slices, we assessed thrombin protein levels using Western Blot analysis. Here, at 10 minutes following OGD recovery, thrombin levels were ~2fold increased (2.07 ± 0.28) compared to control condition (1.10 ± 0.16, n = 12; $p < 0.01$; Fig. 1d). Interestingly, mRNA levels of prothrombin (the thrombin precursor), were also affected by *in vitro* OGD. Prothrombin mRNA-levels were markedly reduced in OGD treated slices compared to controls (control: 1.00 ± 0.13; OGD: 0.42 ± 0.11; 10 minutes following OGD recovery; n = 12; $p < 0.05$). All together the protein and mRNA data may indicate a rapid negative feedback mechanism of increased thrombin levels/activity on prothrombin expression upon OGD *in vitro*. In summary, these experiments confirmed that OGD causes iLTP and that changes in thrombin activity and prothrombin mRNA levels occur in acute slices upon *in vitro* OGD.

Thrombin and PAR1 activation mediate iLTP. In order to assess whether thrombin is involved in iLTP induction, OGD experiments were repeated in the presence of the specific thrombin inhibitor α-NAPAP (1 μM)[12,25]. Indeed, iLTP was impaired in these experiments: EPSP slopes were 1.10 ± 0.06 at 10 minutes following OGD recovery compared to 0.97 ± 0.05 at the baseline (n = 12; $P = 0.52$; Fig.2a).

We then tested whether thrombin could act through the activation of its receptor PAR1 to induce OGD-mediated iLTP[16,17,26]. To this end, another set of slices were exposed to OGD in presence of the selective PAR1 antagonist SCH79797 (1 μM)[12,27]. Under this experimental condition, iLTP was also not evoked (1.06 ± 0.086 at 10 minutes after OGD recovery vs. 1.02 ± 0.06 at the baseline, n = 12; $P = 0.44$, Fig. 2b) indicating that PAR1 activation is required for the induction of iLTP.

To confirm these findings, in a different set of experiments, hippocampal slices prepared from PAR1-KO mice were exposed to OGD. Figure 2C shows that iLTP could not be elicited after OGD in slices lacking PAR1 (n = 12). These experiments demonstrated that iLTP is mediated by PAR1, which can be activated by thrombin following OGD in vitro.

Exogenous thrombin application and pharmacological PAR1 activation occlude the ability to induce iLTP. In order to gain further evidences on the role of thrombin and PAR1 on iLTP induction, we hypothesized that previous exposure of the slice to either thrombin or a specific PAR1 agonist will occlude further

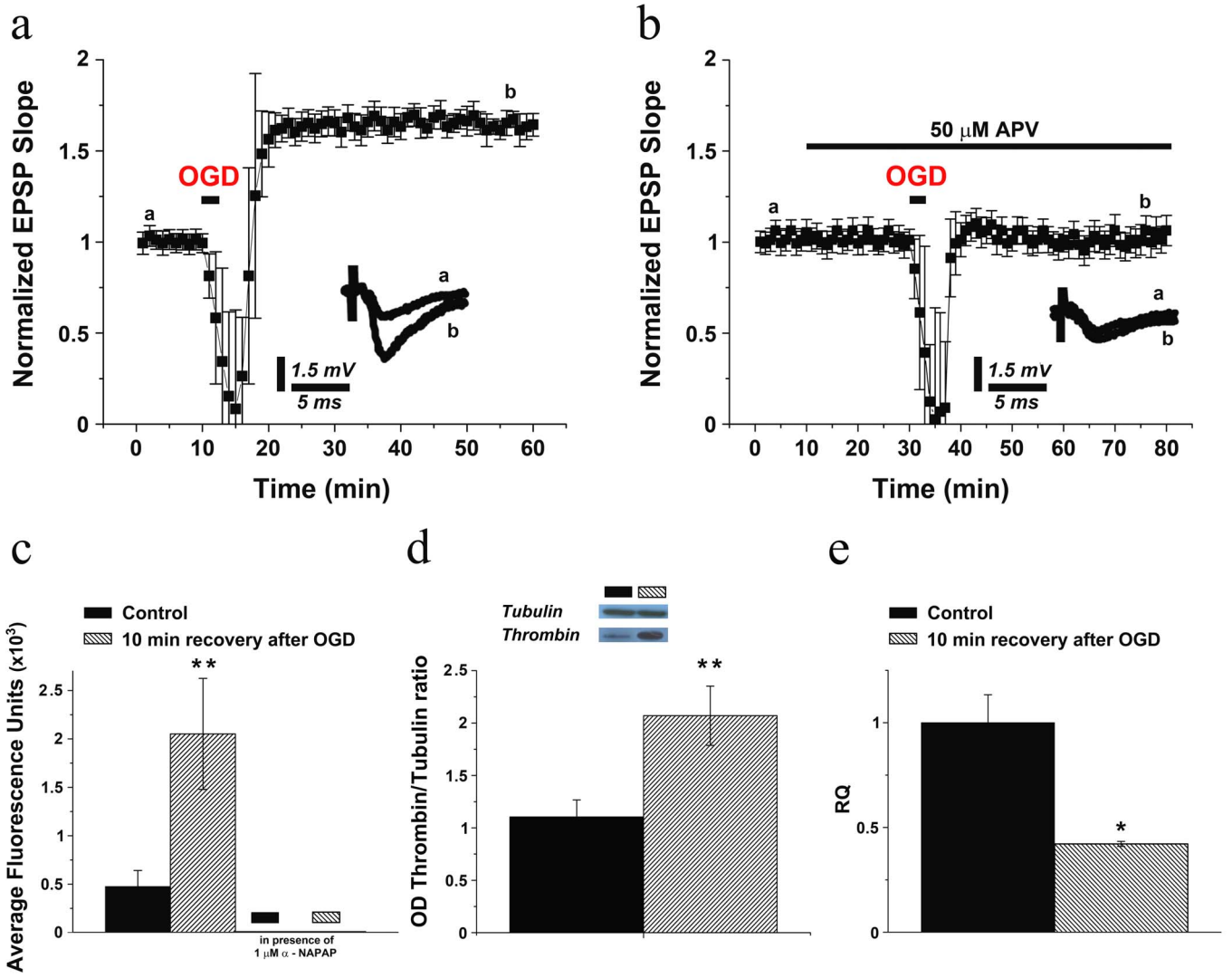

Figure 1 | OGD induces iLTP and increases thrombin activity and protein levels in acute hippocampal slices. (*a*) A brief exposure (3 minutes) to OGD induces iLTP in the hippocampus which is blocked by previous application of 50 μM of APV, an NMDAR antagonist (*b*). Averaged EPSPs are plotted versus time. Representative traces at indicated times (a, b) are shown for each section. In hippocampal slices undergoing OGD, (*c*) thrombin activity and (*d*) concentration are enhanced, while (*e*) prothrombin mRNA is reduced 10 minutes upon OGD recovery. * $p < 0.05$; ** $p < 0.01$.

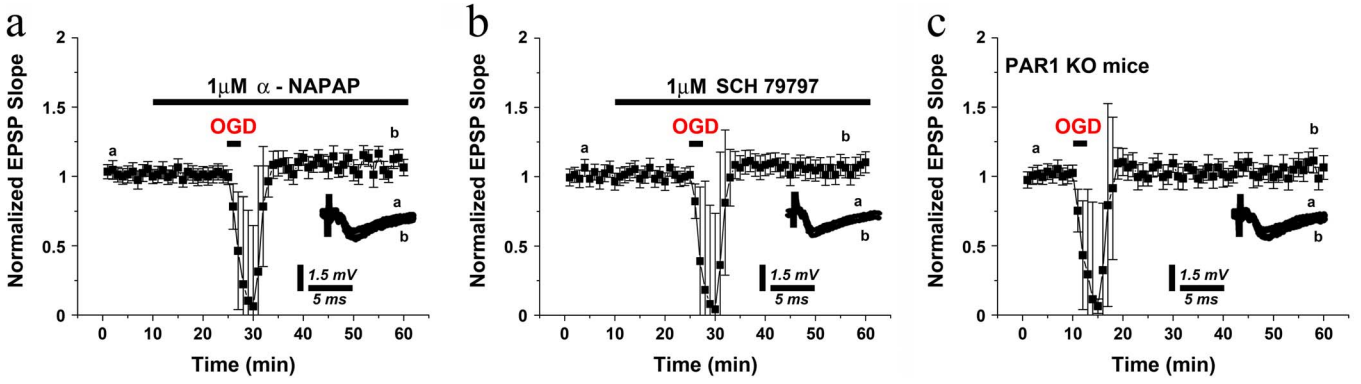

Figure 2 | Blockade of either thrombin or PAR1 fails to induce iLTP in acute hippocampal slices. A brief exposure (3 minutes) to OGD in presence of either (*a*) 1 μM of the thrombin inhibitor α-NAPAP or (*b*) 1 μM of the PAR1 antagonist SCH797977 fails to induce iLTP. (*c*) A brief exposure to OGD (3 minutes) fails to induce iLTP in PAR1$^{-/-}$ mice. Averaged EPSPs are plotted versus time. Representative traces at indicated times (a, b) are shown for each section.

potentiation following OGD, due to the earlier recruitment of the PAR1 signaling pathway. This assumption was tested in an experimental setting using an additional stimulating electrode in order to examine two parallel pathways in the same slice[28]. Exposure to either 1 U thrombin or 1 μM of the specific PAR1 agonist-activating peptide SFLLRN (PAR1-AP, Fig. 3a and 3b) resulted in a slow onset LTP, as shown previously[17]. When potentiation levels reached asymptote, stimulation intensity at one pathway was reduced to the level before drug application and OGD was then applied. In this condition, a transient depression of transmission was still evoked in both pathways following exposure to OGD. However, no iLTP was induced in the previously depotentiated path, indicating that prior exposure to either thrombin or PAR1-AP saturated the ability to induce further iLTP. At the alternate path, EPSP slopes reached the same levels of potentiation previously accomplished (Fig. 3a and 3b). These experiments concluded that thrombin induces iLTP in hippocampal slices exposed to OGD through the activation of PAR1.

Thrombin and PAR1 inhibition restore tetanus induced LTP in OGD exposed hippocampal slices. Since iLTP is considered to be a pathological form of synaptic plasticity, which alters the plastic properties of healthy neuronal networks[4], we reasoned that physiological, i.e., tetanus-induced LTP will be impaired in a network previously exposed to OGD. In order to test this hypothesis, in a two pathway experimental setting, hippocampal slices were once more exposed to OGD. This resulted in iLTP at both pathways recorded in parallel (Fig. 4a). When potentiation levels reached saturation, the stimulation intensity at one pathway was reduced to the level of baseline and a tetanic stimulation (100 Hz; 1 second) was then delivered at this pathway. Strikingly, the tetanus did not evoke LTP (0.993 ± 0.06 at 25 minutes after tetanic stimulation, n = 12, Fig. 4a) under these conditions.

We then blocked thrombin with 1 μM α-NAPAP or PAR1 with 1 μM SCH79797 prior to OGD and tested for the ability to induce LTP using the same experimental approach as described above (Fig. 4b and Fig. 4c). Strikingly, in these experiments not only iLTP was blocked (c.f., Fig. 2), but also the ability to induce LTP after OGD was restored in α-NAPAP (Fig. 4B) or SCH79797 (Fig. 4C) treated slices. In summary, these experiments confirmed that OGD-induced iLTP saturates the ability of neurons to express synaptic plasticity and impairs the establishment of further LTP. These findings confirm the pathological role of iLTP and furthermore suggest that pharmacological inhibition of either thrombin or PAR1, restores physiological synaptic plasticity in OGD neuronal networks.

Discussion

In this manuscript, we investigated the role of thrombin in synaptic plasticity following OGD. Using an *in vitro* model of acute ischemia, we were able to demonstrate that thrombin activity, which is enhanced upon OGD *in vitro*, induces iLTP through the activation of PAR1 and NMDARs (Fig.5). Strikingly, blockade of thrombin and PAR1 signaling inhibited iLTP and restored the ability of neurons to express physiological synaptic plasticity following OGD.

The biological consequences of iLTP for the course of cerebrovascular diseases remain not well understood. On the one hand it has been proposed that iLTP may alter plasticity in healthy neuronal networks[4]. The findings of the present study support this suggestion, since iLTP saturated LTP-generating mechanism in a nonselective manner. Thus, iLTP may influence and therefore hamper the functional recovery from a cerebrovascular trauma: an unspecific saturation of synaptic connectivity by iLTP may alter specific LTP-like plastic processes in the brain serving the acquisition of new "memories" or adaptation to new motor plans after the insult[17]. Whether iLTP contributes to excitotoxicity and neuronal cell death at a later stage remains to be determined. On the other hand, iLTP may act as a protective, i.e., homeostatic mechanism by stabilizing synapses in acute ischemia and maintaining circuit excitability under pathological conditions. We are currently investigating this intriguing hypothesis.

Here, we provide the first experimental evidence that thrombin, whose activity and concentration is known to increase following acute ischemia[10,29], mediates iLTP via PAR1-activation. Previous work has indicated that tissue plasminogen activator (tPA) may be also involved in iLTP induction[4,30]. Certainly, our data do not exclude this possibility as (i) tPA has also been shown to activate PAR1[31] and (ii) thrombin is known to promote tPA release[32]. However, while thrombin has been shown to regulate the early phase of LTP[16,17], tPA seems to be fundamental for sustaining its late phase[33–36], thus implicating thrombin to have a major role in the early phase of iLTP induction. Although our α-NAPAP and thrombin experiments clearly show an important role for thrombin in iLTP, additional

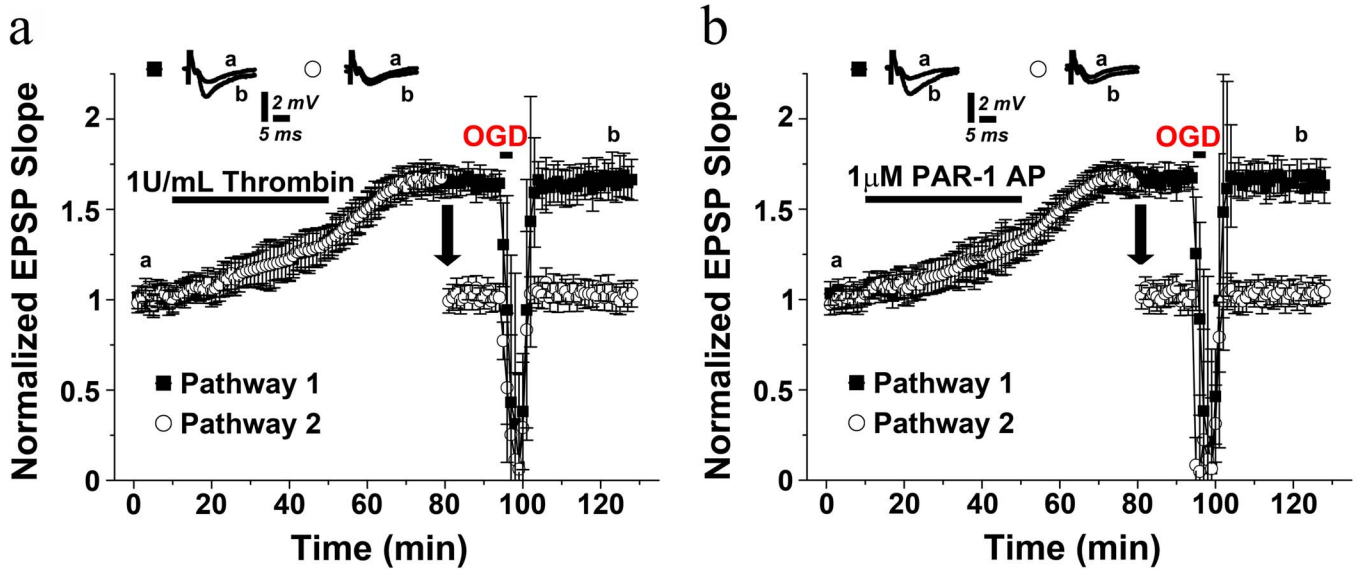

Figure 3 | Previous exposures to thrombin or PAR1-AP occlude the induction of later iLTP. Either (*a*) 1 U thrombin or (*b*) 1 μM PAR1-AP induce a slow onset LTP and saturate the induction of further potentiation upon brief exposure (3 minutes) to OGD. Averaged EPSPs are plotted versus time. Representative traces at indicated times (a, b) are shown for each section.

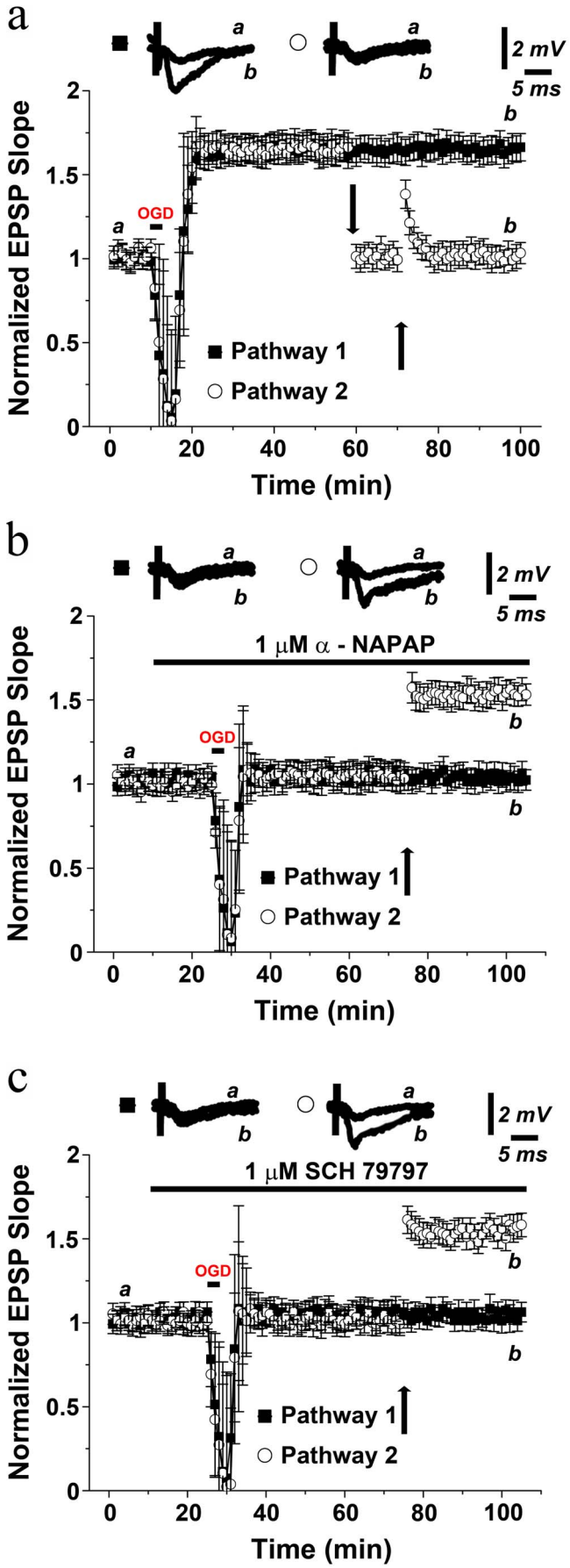

Figure 4 | Either thrombin or PAR1 blockade restore physiological tetanus induced LTP in OGD exposed hippocampal slices. (*a*) A brief exposure (3 minutes) to OGD induces iLTP and blocks stimulus onset LTP: a tetanic stimulation to the same pathway, adjusted to submaximal level (downward arrow), is able to produce a short-term but not a long-term potentiation. (*b*) Blockade of either thrombin or PAR1 through (*b*) 1 μM α-NAPAP or (*c*) 1 μM SCH79797 respectively blocks iLTP and restores tetanus induced LTP. Averaged EPSP are plotted versus time. Representative traces at indicated times (a, b) are shown for each section. Upward arrows indicate the time of HFS.

experiments are required to better understand the complex and yet puzzling interactions between tPA and thrombin in synaptic plasticity.

Recently, thrombin has been shown to play divergent roles in synaptic plasticity depending on its concentrations at the synaptic cleft[12]. While physiological, i.e., low concentrations of thrombin promote the induction of LTP, higher (yet not toxic) concentrations induce a slow onset LTP which saturates the ability of neurons to establish further physiological synaptic plasticity[17], similar to what is observed after the induction of iLTP (this study). Whether OGD-induced iLTP and thrombin-induced slow onset LTP resemble the same mechanisms acting at different time scales remains to be shown. It should be noted, however, that thrombin is a fairly large molecule whose delayed penetration rate in the slices may account for the observed slow onset effects[17]. Instead, under OGD, high concentrations of thrombin may be readily released locally to act at a much faster time scale, as seen in the context of iLTP: endogenous thrombin activity increases rapidly after OGD *in vitro*.

An interesting consequence of our experiments is that thrombin expression and activity can be regulated in the brain under conditions where no blood is present. In acute hippocampal slices both thrombin activity, thrombin protein levels and prothrombin mRNA levels were changed within 10 minutes following OGD. Also we were able to block endogenous thrombin and PAR1-activity pharmacologically in our preparations. This finding has fundamental implications as it shows that thrombin may be a "brain-borne", i.e., neural molecule whose expression is tightly regulated in specific conditions, such as early ischemic events. In a later stage, upon blood brain barrier (BBB) breakdown caused by prolonged ischemia, additional, blood-derived thrombin may enter into the brain and trigger severe events such as seizures and cell death[37–41]. While we have shown that thrombin may be actively regulated in the brain, at this time we have

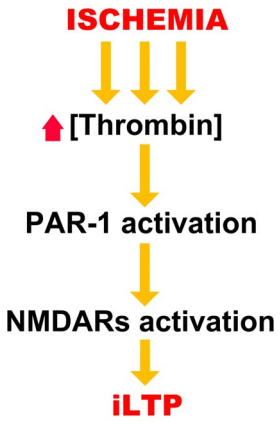

Figure 5 | Thrombin induces iLTP. Upon ischemia, the increased thrombin activity leads to the activation of PAR1 and NMDARs thus causing iLTP.

not focused on the cellular source of thrombin and the molecular machinery responsible for its formation and release. It has been already known for decades that thrombin is physiologically expressed in the brain at low concentration levels[42–44]. Though additional experiments are now needed in order to understand how thrombin expression/activity is regulated in the brain.

Thrombin is known to act via PAR1, which is expressed both in neurons and astrocytes[31,45]. Our experiments show that PAR1-KO animals lack iLTP. These animals have been reported to have normal synaptic transmission, slight deficits of hippocampal functions and reduced levels of late LTP; however they have normal NMDARs function[46]. This latter evidence excludes that the lack of iLTP in PAR1-KO hippocampal slices is due to impaired NMDAR transmission. Remarkably, experimental evidence has been provided that PAR1-KO animals show enhanced neuroprotection following ischemic stroke[47,48]. Thus, inhibition of thrombin and/or PAR1 may be a potential target for developing novel therapeutic strategies in order to enhance both synaptic plasticity (e.g., by blocking iLTP) and neuronal survival following an ischemic insult.

In conclusion, we show that thrombin, whose activity rises in brain slices upon OGD, leads to iLTP through activation of PAR1 and consequent activation of NMDARs (Fig. 5). These data add novel, fundamental information on the early changes occurring at excitatory synapses after ischemia and hint to novel strategies to restore function after stroke.

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