Neuronal protease-activated receptor 1 drives synaptic retrograde signaling mediated by the endocannabinoid 2-arachidonoylglycerol

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Neuronal protease-activated receptor 1 drives synaptic retrograde signaling mediated by the endocannabinoid 2-arachidonoylglycerol

Abbreviated title: Roles of PAR1 in Endocannabinoid Signaling

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Abstract

Protease-activated receptor 1 (PAR1) is a member of the G-protein coupled receptors that are proteolytically activated by serine proteases. Recent studies suggest a definite contribution of PAR1 to brain functions including learning and memory. However, cellular mechanisms by which PAR1 activation influences neuronal activity are not well understood. Here we show that PAR1 activation drives retrograde endocannabinoid signaling and thereby regulates synaptic transmission. In cultured hippocampal neurons from rat, PAR1 activation by thrombin or PAR1-specific peptide agonists transiently suppressed inhibitory transmission at cannabinoid-sensitive, but not cannabinoid-insensitive, synapses. The PAR1-induced suppression of synaptic transmission was accompanied by an increase in paired-pulse ratio, and was blocked by a cannabinoid CB_1 receptor antagonist. The PAR1-induced suppression was blocked by pharmacological inhibition of postsynaptic diacylglycerol lipase (DGL), a key enzyme for biosynthesis of the major endocannabinoid 2-arachidonoylglycerol (2-AG), and was absent in knockout mice lacking the α isoform of DGL (DGLα). The PAR1-induced IPSC suppression remained intact under the blockade of metabotropic glutamate receptors, and was largely resistant to the treatment that blocked Ca^{2+} elevation in glial cells following PAR1 activation, which exclude the major contribution of glial PAR1 in IPSC suppression. We conclude that activation of neuronal PAR1 triggers retrograde signaling mediated by 2-AG, which activates presynaptic CB_1 receptors and suppresses transmitter release at hippocampal inhibitory synapses.

Introduction

Protease-activated receptors (PARs) are members of seven-transmembrane domain G-protein coupled receptors and consists of four subtypes (PAR1-PAR4) (Ossovskaya and Bunnett, 2004). Activation of PARs is initiated by a unique mechanism that protease cleaves a specific site within extracellular N terminus region of the receptor. This cleavage reveals a new N terminus, which acts as a tethered ligand and then binds and activates the receptor. PARs are abundantly expressed in the brain, and have been shown to play various roles in pathophysiological conditions such as neurogenic inflammation, neurodegeneration and neuroprotection (Ossovskaya and Bunnett, 2004; Luo et al., 2007).

PARs may also contribute to neural functions under physiological conditions. PAR1-deficient mice show defects in passive avoidance task, cued fear conditioning (Almonte et al., 2007) and nicotine-induced place preference (Nagai et al., 2006). At cellular levels, PAR1 activation influences synaptic transmission. PAR1 activation potentiated NMDA receptor currents (Gingrich et al., 2000; Mannaioni et al., 2008) and induced long-term potentiation (LTP) of excitatory transmission (Maggio et al., 2008) in CA1 pyramidal neurons of hippocampal slices, and enhanced spontaneous release of glutamate onto substantia gelatinosa neurons in spinal cord slices (Fujita et al., 2009). As to the mechanisms, several studies have demonstrated that PAR agonists activate astrocytic PAR1, rather than neuronal PAR1, induce glutamate release from astrocytes, and thereby activate neuronal NMDA receptors in hippocampal acute slices (Lee et al., 2007; Mannaioni et al., 2008; Shigetomi et al., 2008) and hippocampal coculture preparations (Lee et al., 2007). *In situ* hybridization studies suggest that PAR1 is expressed in both astrocytes and neurons including hippocampal pyramidal and granule cells (Weinstein et al., 1995; Niclou et al., 1998). Our knowledge about roles of neuronal PAR1 in the regulation of synapic transmission is, however, rather limited, especially at inhibitory synapses.

Endocannabinoid-mediated retrograde modulation of synaptic transmission plays important roles in various brain functions (Heifets and Castillo, 2009; Kano et al., 2009). Endocannabinoids are released from postsynaptic neurons and retrogradely activate presynaptic CB_1 receptors, which lead to suppression of transmitter release. Endocannabinoid release is induced by postsynaptic depolarization (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) and/or activation of several types of $G_{q/11}$-coupled receptors including group I metabotropic glutamate receptors (I-mGluRs) (Maejima et al., 2001). Activation of phospholipase Cβ (PLCβ) through these receptors produces diacylglycerol (DG), which is then hydrolyzed by DG lipase α (DGLα) and yields the endocannabinoid 2-arachidonoylglycerol (2-AG) (Hashimotodani et al., 2005; Maejima et al., 2005; Gao et al., 2010; Tanimura et al., 2010). Importantly, PAR1 activates $G_{q/11}$ protein and stimulates phosphoinositide hydrolysis via PLCβ (Ossovskaya and Bunnett, 2004; McLaughlin et al., 2005). Therefore, it is likely that PAR1 activation can drive endocannabinoid signaling. This possibility, however, has never been tested electrophysiologically. In the present study, we used cannabinoid-sensitive inhibitory synapses of cultured hippocampal neurons, and demonstrated for the first time that neuronal PAR1 activation can modulate the synaptic transmission through the endocannabinoid 2-AG signaling.

Materials and Methods

Cell culture

All experiments were performed according to the guidelines laid down by the animal welfare committees of the University of Tokyo, Kanazawa and Niigata Universities. Cultured hippocampal neurons were prepared from newborn Sprague-Dawley rats, PAR1 knockout mice (The Jackson Laboratory, Bar Harbor, ME) (Connolly et al., 1996), DGLα knockout mice (Tanimura et al., 2010) and their wild-type littermates as described previously (Ohno-Shosaku et al., 2001). Animals with both sexes were used.

Briefly, cells were mechanically dissociated from the hippocampi and plated onto poly-L-ornithine-coated plastic dishes for electrophysiological experiments or low-fluorescence plastic films (Sumilon MS-92132, Sumitomo, Tokyo, Japan) for Ca^{2+} imaging. The cultures were kept at 36 °C in 5 % CO_2 for 12-15 days before use.

Electrophysiology

Double whole-cell recordings were performed from cultured hippocampal neurons using a patch-clamp amplifier (EPC10/2; HEKA Electronik, Lambrecht/Pfalz, Germany). Each neuron of a pair was voltage clamped at -80 mV using a patch pipette (3-5 MΩ) filled with the internal solution with the following compositions (in mM): 130 K-gluconate, 15 KCl, 10 HEPES, 0.2 EGTA, 6 $MgCl_2$, 5 Na_2ATP and 0.2 Na_2GTP (pH 7.3, adjusted with KOH). For the experiments shown in Figure 3E, we used the internal solution with the following compositions for postsynaptic neurons (in mM): 109.6 (for pCa 6) or 121.8 (for pCa 9) K-gluconate, 15 KCl, 10 HEPES, 10 BAPTA, 8.47 (for pCa 6) or 0.0549 (for pCa 9) $CaCl_2$, 5 $MgCl_2$, 5 Na_2ATP and 0.2 Na_2GTP (pH 7.3, adjusted with KOH) (Hashimotodani et al., 2005). One neuron was stimulated by applying positive voltage pulses (to 0 mV, 2 ms) at 0.5 Hz or 0.25 Hz, and IPSCs were measured from the other neuron. The external solution contained (in mM): 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose and 1 kynurenic acid (pH 7.3, adjusted with NaOH). The recording chamber was perfused with the external solution at a flow rate of 1-3 ml/min. Our culture preparations contain cannabinoid-sensitive and -insensitive inhibitory neurons (Ohno-Shosaku et al., 2001). The cannabinoid sensitivity of IPSCs was determined by triggering depolarization-induced suppression of inhibition (DSI), a form of endocannabinoid-mediated short-term depression (Alger, 2002). We defined cannabinoid-sensitive and -insensitive IPSCs by DSI-positive (> 30% of suppression) and DSI-negative (< 10% of suppression) IPSCs, respectively. PAR1 activators (thrombin, TFLLR, SFLLRN) were locally applied for 1 min through a

capillary tube (250 μm inner diameter) located near the recorded neurons using a perfusion valve controller (VC-6M; Warner Instruments, Hamden, CT). All experiments were performed at room temperature.

Ca^{2+} imaging

Hippocampal cultures on the films were incubated with 5 μM fura-2 AM (Dojindo, Kumamoto, Japan) at 36 °C for 15 min and then transferred to a glass-based dish mounted with recording chamber and perfused with the external solution (see above); 1 mM kynurenic acid was replaced with 0.1 μM tetrodotoxin. Fluorescence signals for excitation of 340 nm and 380 nm were measured at 0.2 Hz by using an imaging system (Acquacosmos, Hamamatsu Photonics, Hamamatsu, Japan) mounted on an inverted microscope (IX70, Olympus, Tokyo, Japan).

Drugs

TFLLR-NH_2, AM251, DHPG, MPEP, LY367385 and thapsigargin were purchased from Tocris Cookson (Bristol, UK). Thrombin, SFLLRN and tetrahydrolipstatin (THL) were purchased from Sigma-Aldrich (St. Louis, MO). LY367385 was dissolved in NaOH-containing water as a stock solution. TFLLR, SFLLRN, thrombin and DHPG were dissolved in water. Other drugs were dissolved in DMSO.

Data analysis

In each experiment, amplitudes of 10 consecutive IPSCs during application of receptor activators were averaged and normalized to the control values before application. Statistical significance was assessed by Student's *t*-test (Figs. 1, 2) or Mann-Whitney U-test (Fig. 3). One, two, and three asterisks indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. All data are presented as mean ± SEM.

Results

Activation of PAR1 induces IPSC suppression

We first examined effects of PAR1 activation on hippocampal IPSCs, using an endogenous PAR activator, thrombin, and two PAR1-selective peptide agonists, TFLLR and SFLLRN. If PAR1 triggers endocannabinoid release, cannabinoid-sensitive IPSCs (see Materials and Methods) would be influenced selectively. As expected, local application of thrombin transiently suppressed cannabinoid-sensitive IPSCs (DSI-positive) (Fig. 1A), but not cannabinoid-insensitive IPSCs (DSI-negative) (Fig. 1B). In the following experiments, we used only the cannabinoid-sensitive IPSCs. In most cases, suppression was recovered gradually from its peak even in the presence of thrombin. When thrombin application was repeated, the second application failed to suppress IPSCs (Fig. 1D, F), which can be explained by receptor desensitization (Kawabata et al., 1999). Similar suppressing effects on cannabinoid-sensitive IPSCs were observed with TFLLR (Fig. 1C) and SFLLRN (29.0 ± 6.0 % of control). When TFLLR was applied repeatedly, the second application failed to suppress IPSCs (Fig. 1E, F). When TFLLR and thrombin were applied sequentially, IPSCs were suppressed only at the first application irrespective of the order (Fig. 1G-I), indicating the involvement of the same receptor. The involvement of PAR1 was further confirmed by the results showing that TFLLR-induced IPSC suppression was absent in PAR1 knockout mice (Fig. 1J-L). The IPSC suppression induced by thrombin or TFLLR was accompanied by an increase in paired-pulse ratio (Fig. 1M), which is generally used as a marker of presynaptic change. These results indicate that PAR1 activation induces presynaptic suppression of inhibitory transmission at cannabinoid-sensitive synapses.

Endocannabinoid signaling is involved in PAR1-mediated IPSC suppression

We next examined whether the PAR1-driven suppression of IPSCs is mediated by endocannabinoids. The IPSC suppressions induced by thrombin and TFLLR were both

abolished by the treatment with AM251 (Fig. 2A, B, E, F). Recent studies have revealed that 2-AG produced by DGLα mediates retrograde synaptic suppression (Gao et al., 2010; Tanimura et al., 2010). Therefore, we tested whether the PAR1-driven suppression of IPSCs is dependent on DGLα, by using pharmacological and genetic tools. Treatment with a DGL inhibitor, THL, completely blocked both thrombin and TFLLR-induced IPSC suppression (Fig. 2C-F). In hippocampal neurons prepared from DGLα knockout mice, TFLLR failed to suppress IPSCs (Fig. 2G-I). From these results, we conclude that PAR1 activation leads to production and release of 2-AG in a DGLα-dependent manner and induces suppression of GABA release through activation of presynaptic CB_1 receptors.

2-AG is produced and released from postsynaptic neurons upon PAR1 activation

We then examined whether 2-AG was produced within postsynaptic neurons upon PAR1 activation, by applying the DGL inhibitor THL to the postsynaptic neurons through patch pipettes. Effectiveness of postsynaptically-applied THL was estimated by monitoring DHPG (I-mGluR agonist)-induced and depolarization-induced IPSC suppressions (Fig. 2J), both of which were previously demonstrated to be blocked by bath applied THL in our preparations (Hashimotodani et al., 2007b; Hashimotodani et al., 2008). When THL was applied postsynaptically, thrombin and TFLLR-induced IPSC suppressions were reduced by similar degrees to DHPG- and depolarization-induced suppressions (Fig. 2J). These results clearly indicate that the IPSC suppression is mediated by 2AG that is produced and released from postsynaptic neurons upon PAR1 activation.

Neuronal PAR1 is responsible for generation of endocannabinoid signaling

A simple model for PAR1-driven 2-AG release is that PAR1 activation and 2-AG production occur in the same postsynaptic neuron. However, it is also possible that

PAR1 activation and 2-AG production occur in separate cells. Recent studies have demonstrated that astrocytes are able to release several gliotransmitters including glutamate, when cytosolic Ca^{2+} concentration is elevated (Perea et al., 2009). Therefore, it is possible that activation of astrocytic PAR1 causes Ca^{2+} elevation and thereby induces glutamate release, which then activates neuronal I-mGluRs (mGluR1 and /or mGluR5) and initiates 2AG production through I-mGluR-PLCβ-DGLα pathway within the postsynaptic neuron. To test this possibility, effects of combined treatment with the mGluR1 antagonist LY367385 and the mGluR5 antagonist MPEP on PAR1-driven IPSC suppression were examined. This treatment blocked the IPSC suppression induced by the I-mGluR agonist DHPG, but failed to block thrombin- and TFLLR-induced suppressions of IPSCs (Fig. 3A).

To test whether gliotransmitters other than glutamate are involved, we treated culture preparations with thapsigargin to deplete Ca^{2+} stores, which is expected to block Ca^{2+} elevation and gliotransmitter release (Mothet et al., 2005). We confirmed that Ca^{2+} transients triggered by TFLLR application were completely blocked in thapsigargin-treated astrocytes (Fig. 3B, C). This treatment attenuated the TFLLR-induced suppression of IPSCs, but left a large part of suppression intact (Fig. 3D). This thapsigargin-resistant component of IPSC suppression should be independent of gliotransmitters, and thus dependent on neuronal PAR1 rather than astrocytic PAR1. As to the thapsigargin-sensitive component, there are two possibilities. This component may be dependent on astrocytic PAR1 and mediated by gliotransmitters. Alternatively, this component may also be dependent on neuronal PAR1, but sensitive to thapsigargin, because neuronal PAR1 activation induces local Ca^{2+} elevation, which can enhance 2-AG production. The endocannabinoid release driven by $G_{q/11}$-coupled receptors such as I-mGluR and M_1/M_3 receptors has been demonstrated to be dependent on basal Ca^{2+} concentration and enhanced by postsynaptic Ca^{2+} elevation (Hashimotodani et al., 2007a). To examine the Ca^{2+} dependence of PAR1-driven 2-AG release, we dialyzed

thapsigargin-treated postsynaptic neurons with either pCa 6 or pCa 9 pipette solution (Hashimotodani et al., 2005). The TFLLR-induced IPSC suppression was more prominent in the neurons filled with the pCa 6 solution (Fig. 3E). Thus, the thapsigargin-sensitive component of IPSC suppression is likely to include a neuronal PAR1-dependent component. Taken together, we concluded that a large component of 2-AG release, if not all, was gliotransmitter-independent and neuronal PAR1-dependent.

Discussion

In this study, we have shown for the first time that activation of neuronal PAR1 triggers retrograde 2-AG signaling. This PAR1-driven 2-AG signaling leads to suppression of inhibitory synaptic transmission through presynaptic CB_1 receptors in hippocampal neurons. Because PAR1 and CB_1 are distributed widely in the brain, this PAR1-CB_1 coupling is likely to be functional, and contribute to synaptic modulation throughout the brain, similarly to other endocannabinoid-producing receptors including glutamate (I-mGluR), acetylcholine (M_1/M_3), serotonin (5-HT_2), oxytocin, orexin, and glucocorticoid receptors (Maejima et al., 2001; Kim et al., 2002; Haj-Dahmane and Shen, 2005; Best and Regehr, 2008; Kano et al., 2009).

Our results are supported by previous biochemical, anatomical and Ca^{2+}-imaging studies. A biochemical study showed effects of thrombin on endocannabinoid levels in dorsal root ganglia (DRG) neurons (Vellani et al., 2008). The treatment of rat cultured DRG neurons with 100 nM thrombin elevated the levels of two major endocannabinoids, anandamide and 2-AG. The authors proposed a model in which PAR-PLCβ-DGLα pathways mediate thrombin-induced 2-AG production. It has been reported that rat cultured hippocampal neurons exhibit PAR1-immnoreactivtiy and expression of mRNA (Gorbacheva et al., 2009). Furthermore, the study using Ca^{2+}-imaging has demonstrated the presence of functional PAR1 in cultured hippocampal neurons (Bushell et al., 2006). Because neuronal PAR1 expression has

been also reported in brain slices (Weinstein et al., 1995; Niclou et al., 1998), we expect that neuronal PAR1-driven 2-AG signaling is functional also *in vivo*. However, cultured neurons could be different from the neurons in vivo in some aspects and we cannot exclude the possibility that PAR1 is up-regulated under culture conditions. Further studies with slice preparations are necessary to understand the physiological significance of the PAR1-driven 2-AG signaling.

Previous studies with hippocampal slices demonstrate that PAR1 activation positively regulates neuronal excitatory synaptic activity (Gingrich et al., 2000; Lee et al., 2007; Maggio et al., 2008; Mannaioni et al., 2008). To our best knowledge, there are no studies reporting PAR1-induced suppression of excitatory transmission. Considering that hippocampal excitatory transmission is much less sensitive to cannabinoids (Ohno-Shosaku et al., 2002), it is possible that 2-AG signaling triggered by PAR1 activation is too weak to cause a detectable change in the excitatory transmission. We showed that PAR1-driven 2-AG signaling was shown to be independent of I-mGluR, indicating that PAR1-induced glutamate release from astrocytes (Lee et al., 2007) contributes little to 2-AG generation. The amount of glutamate released from astrocytes might be too small to trigger 2-AG synthesis in neurons. It is also possible that glia-neuron crosstalk was disconnected in our culture preparations, although a previous study clearly demonstrated that the crosstalk was intact even in culture (Lee et al., 2007).

In the hippocampus, PAR1 activators could exert opposite actions on excitatory and inhibitory synaptic transmissions. The potentiation of excitation is mediated by astrocytic PAR1 and gliotransmitters (Lee et al., 2007; Mannaioni et al., 2008), and the suppression of inhibition is mediated by neuronal PAR1 and endocannabinoids. If these two pathways are driven simultaneously, the excitatory signal is synergistically potentiated and LTP induction may be facilitated. The finding that PAR1 triggers 2-AG signaling provides a new insight into the roles of PAR1 under not only physiological but

also pathological conditions. The endocannabinoid system has also been reported to be associated with neurodegeneration and neuroprotection (Fowler et al., 2010). It will be of great interest to investigate whether PAR1-driven 2-AG signaling contributes to the previously reported roles of PAR1 in the CNS.

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Figure legends

Figure 1

Thrombin and PAR1 agonists induce transient suppression of IPSCs.

A-C, (top) Representative IPSC traces before (left) and during (right) application of thrombin (1 U/ml, red) or TFLLR (10 μM, blue). Each trace is the average of five IPSCs. (bottom) Normalized IPSC time course during application of PAR1 activators. Data were obtained from cannabinoid-sensitive (A, C) and cannabinoid-insensitive (B) IPSCs. (Insets) Representative data showing positive (A) and negative (B) DSI responses. A depolarizing pulse (5 s, 0 mV) was applied to the postsynaptic neuron at time zero. ***D-F,*** Representative experiments (D, E) and summary bar graph (F) for the effects of repeated application of thrombin or TFLLR. Two IPSC traces acquired before (black) and during application of thrombin (red) or TFLLR (blue) are superimposed in each sample record in insets. ***G-I,*** Effects of sequential application of thrombin (Thr) and TFLLR (TF), illustrated similarly to D-F. ***J-L,*** Effects of TFLLR (10 μM) on IPSCs in WT and PAR1-KO mice. Two IPSC traces acquired before (black) and during (pink) application of thrombin or TFLLR are superimposed in each sample record in insets. ***M,*** (left) Examples of IPSC traces in response to paired stimuli (60 ms interval) before (black) and during application of thrombin (red) or TFLLR (blue). (right) Averaged data for the increase in paired-pulse ratio by thrombin and TFLLR. Numbers of tested cells are indicated in parenthesis in F, I, L, M and the following figures.

Figure 2

Involvement of endocannabinoid signaling in PAR1-induced IPSC suppression.

A-D, Representative data showing effects of AM251 (0.3 μM, ***A, B***) and THL (5 μM, ***C, D***) on IPSC suppression induced by thrombin (1U/ml) or TFLLR (10 μM). Representative IPSC traces acquired before (left) and during application of thrombin or TFLLR (right) are shown in insets of each panel. ***E, F,*** Summary graph showing normalized IPSC amplitudes during application of thrombin (***E***) or TFLLR (***F***) in the presence of AM251 and THL. ***G-I,*** TFLLR-induced IPSC suppression is absent in DGLα-KO mice. Data are illustrated similarly to Figure 1J-L. ***J,*** Intracellular application of THL (10 μM) to a postsynaptic neuron greatly reduced IPSC suppressions by thrombin (1U/ml), TFLLR (10 μM), DHPG (20 μM), and postsynaptic depolarization (5 s) to the similar extents. Filled and open columns represent normalized IPSC amplitudes for control and THL injected neurons.

Figure 3

Neuronal PAR1, rather than glial PAR1, is responsible for generation of 2-AG signaling. ***A,*** Normalized IPSC amplitudes during application of thrombin (1U/ml), TFLLR (10 μM) and DHPG (20 μM) in the presence (open columns) or absence (filled columns) of a mixture of MPEP (5 μM) and LY367385 (100 μM). ***B, C,*** Representative data (***B***) and summary graph (***C***) showing complete suppression of TFLLR-induced Ca^{2+} elevation in glial cells by thapsigargin (1 μM). ***D,*** Normalized IPSC amplitudes during application of TFLLR (10 μM) after treatment with thapsigargin (1 μM) for 1 h (open column). The control data without thapsigargin-treatment (filled column, presented in Fig. 2F) are shown for comparison. ***E,*** TFLLR-induced IPSC suppression was dependent on postsynaptic Ca^{2+} concentration. After treatment with thapsigargin (1 μM, 1 h), TFLLR (10 μM) was applied to the postsynaptic neurons dialyzed with two different pipette solutions, pCa 6 (n = 7) and pCa 9 (n = 8). Results from non-treated neurons (Fig. 1C) were superimposed (gray triangle).

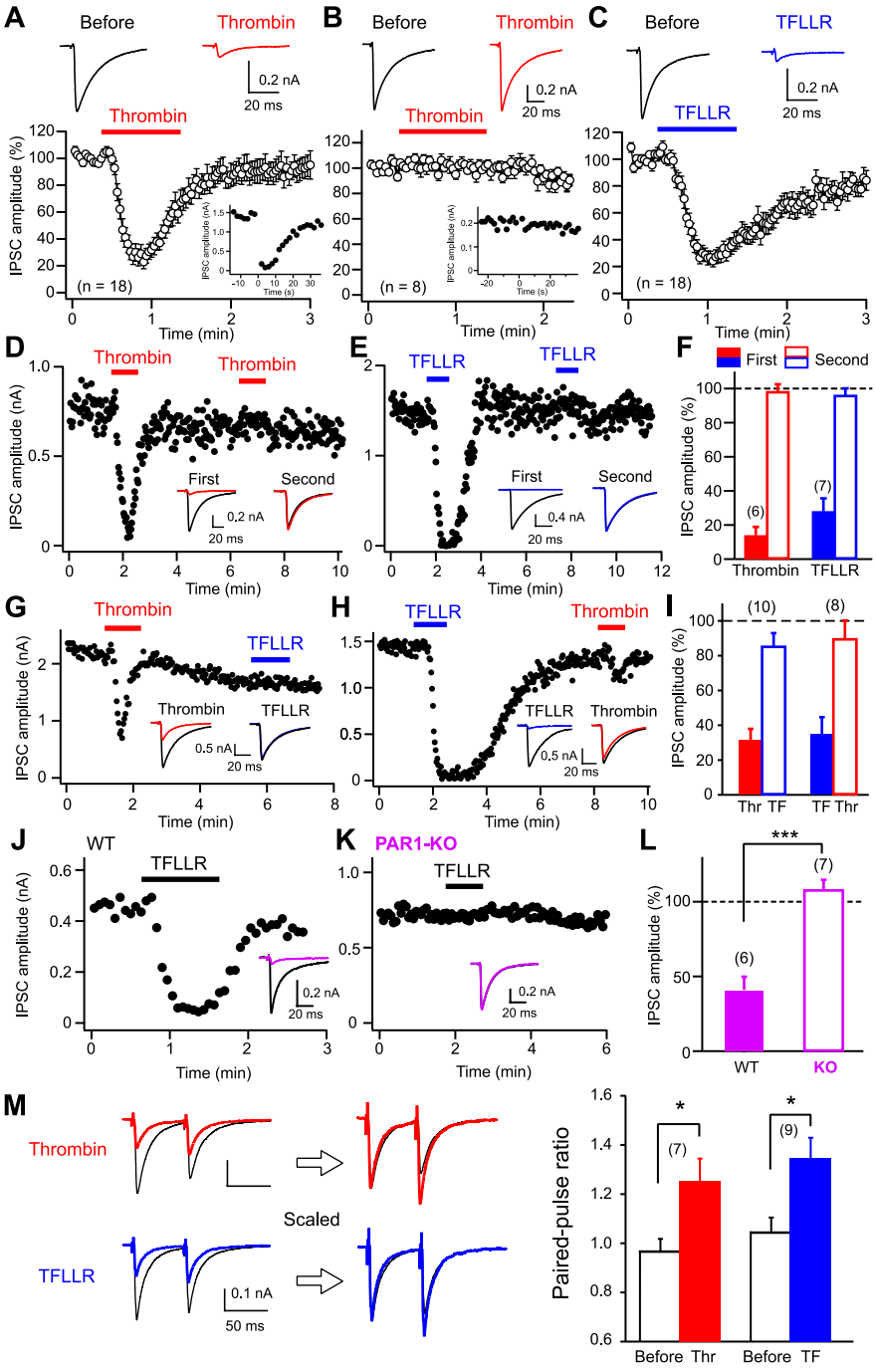

Figure 1

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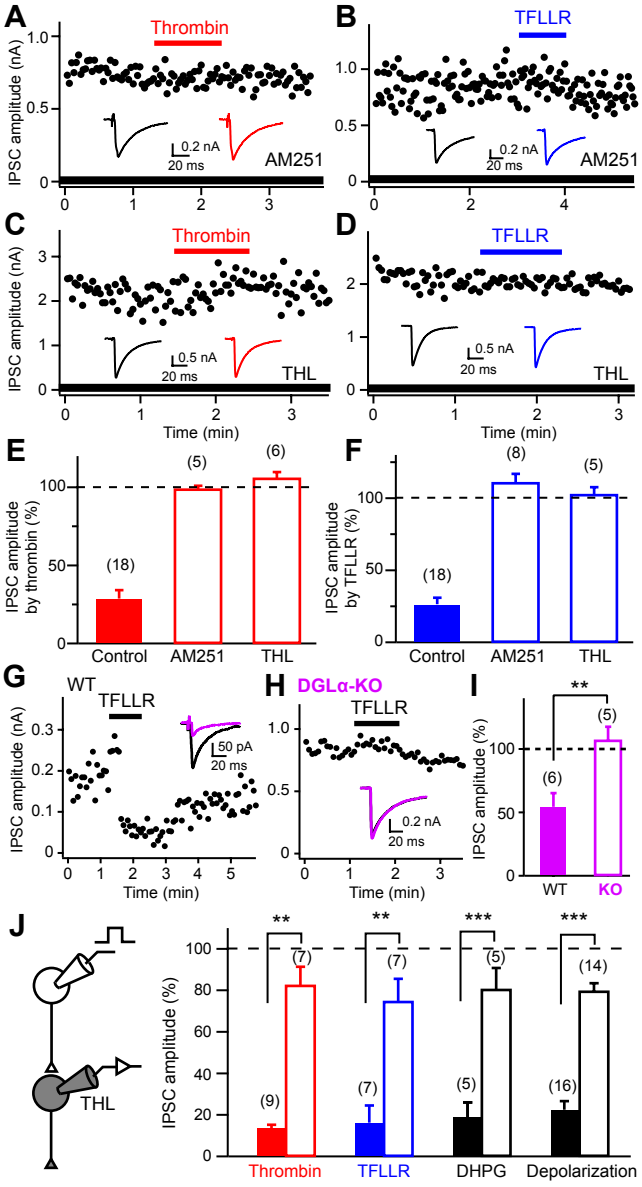

Figure 2

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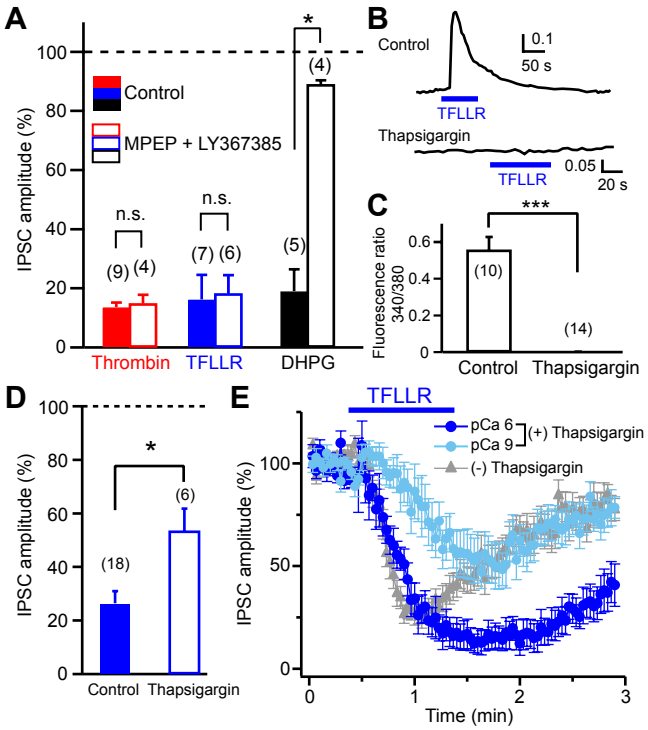

Figure 3

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