Facilitatory effect of docosahexaenoic acid on *N*-methyl-D-aspartate response in pyramidal neurones of rat cerebral cortex

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1. The effect of docosahexaenoic acid (DHA) on *N*-methyl-D-aspartic acid (NMDA) responses in the presence of glycine was investigated in pyramidal neurones acutely dissociated from rat cerebral cortex in whole-cell and single channel configurations.

2. DHA potentiated the NMDA-induced response but reduced the non-NMDA (kainate-induced) response in a concentration-dependent manner at a holding potential of −60 mV under voltage-clamp conditions.

3. Arachidonic acid (AA) also potentiated the NMDA-induced response in a manner similar to DHA. Oleic acid caused a slight potentiation. However, other polyunsaturated and saturated fatty acids had no such effects.

4. The facilitatory action of DHA on the NMDA-induced response was not affected by adding inhibitors of cyclo-oxygenase, lipoxygenase or phospholipase A_2, suggesting that DHA may exert its facilitatory effect directly on the NMDA receptor.

5. The facilitatory action of DHA was observed in the presence of a saturating dose of NMDA. Moreover, a detailed analysis of the NMDA receptor-operated single channel currents revealed that, in the presence of DHA, the open probability of the channel increased without changing the conductance, indicating that DHA may act by binding directly to a novel site on the NMDA receptor or by altering the lipid environment of the NMDA receptor and thereby potentiating the response to NMDA.

6. The results are discussed in terms of the possibility that DHA may play an important role in the genesis of long-term potentiation, at least that involving the activation of NMDA receptors.

Much attention has been focused on the NMDA receptor because of its role in long-term potentiation (LTP). The induction of LTP requires the activation of NMDA receptors, resulting in Ca^{2+} influx into postsynaptic neurones in the CA1 region of the hippocampus (Kauer, Malenka & Nicoll, 1988) and neocortex (Artola & Singer, 1987) of rats. This type of LTP is associated with a sustained increase in transmitter release from the presynaptic terminal (Bliss, Douglas, Errington & Lynch, 1986), implying the existence of a retrograde messenger. The most attractive candidate has been arachidonic acid (AA) and its metabolites (Williams & Bliss, 1988). In a recent study, AA directly potentiated the current through NMDA receptor–channel complexes in rat cerebellar granule cells (Miller, Sarantis, Traynelis & Attwell, 1992).

Docosahexaenoic acid (DHA) is one of the major long-chain polyunsaturated fatty acids normally occurring in mammals and fishes (Crawford, Casperd & Sinclair, 1976). It is found in the highest concentration in the brain and retina. The concentration ratios of DHA to AA are 1·4 in rat brain (Dhopeshwarkar & Subramanian, 1975) and 2·4 in rat retina (Tinoco, Babcock, Hincenberg, Medwadowski & Miljanich, 1978). DHA is localized in the 2-position of membrane phospholipids, especially in phosphatidyl-ethanolamine (Hadjiagapiou & Spector, 1987) and may exert considerable influence on the membrane fluidity and functional properties of integral proteins (Stubbs & Smith, 1984). Recently, it has become apparent that a deficiency in DHA is associated with a loss of discriminant learning ability and visual acuity (Neuringer, Connor, Lin, Barstad & Luck, 1986; Fujimoto *et al.* 1989). Moreover, patients with Zellweger's syndrome (Martinez, 1990) or Alzheimer's disease (Söderberg, Edlunk, Kristensson & Dallner, 1991) show extremely low levels of DHA in their brains.

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However, fewer data are available on the action of DHA in membrane phospholipids, and little is known about the mechanism by which DHA affects several functions after release by phospholipase A_2. In the present study, therefore, the action of DHA on the functional responses to NMDA was examined in pyramidal neurones acutely isolated from rat cerebral cortex in whole-cell and single channel modes under voltage-clamp conditions.

METHODS

Preparation

Pyramidal neurones in the rat cerebral cortex were freshly dissociated as reported previously (Ito, Wakamori & Akaike, 1991) with some modifications. Briefly, 1- to 2-week-old Wistar rats were decapitated under ether anaesthesia. Cortical slices (350 μm thick) were obtained by a vibratome tissue microslicer (DTK-1000, DSK, Kyoto, Japan), and pre-incubated in incubation solution saturated with 95% O_2–5% CO_2 for 40 min at room temperature (22–25 °C). Then the slices were treated enzymatically in an oxygenated standard external solution with 0·015% pronase for 15 min at 31 °C and successively in another standard solution containing 0·015% thermolysin to stop the pronase activity under the same conditions. After the enzyme treatments the slices were thoroughly washed with Ca^{2+}-free ethyleneglycol-bis-*N,N,N',N'*-tetraacetic acid (EGTA) external solution and incubated for another 15 min in standard solution. Thereafter, the cortical pyramidal neurones were dissociated mechanically with fine fire-polished glass Pasteur pipettes in a small plastic culture dish (Falcon) filled with the standard solution under the visual guidance of a phase contrast microscope (Nikon, TMS-1). Dissociated cortical pyramidal neurones adhered to the bottom of the dish within 30 min, allowing electrophysiological studies. The neurones that retained their original morphological features such as the dendritic processes were used throughout the experiment.

Electrical measurement

Electrical recordings were performed in the whole-cell and outside-out excised modes (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) at room temperature (22–25 °C). Recording micropipettes (capillary tubes of 1·5 mm outer diameter, Narishige, Tokyo, Japan) were pulled in two stages using a vertical puller (PB-7, Narishige). The resistance between the patch pipette filled with the internal solution and the reference electrode was 4–8 MΩ. The membrane currents were measured by a patch-clamp amplifier (CEZ-2300, Nihon Kohden, Tokyo, Japan). Signals were filtered with a low-pass filter at −3 dB (FV-665, NF Electronic Instruments, Tokyo, Japan) with a cut-off frequency of 1 kHz (5 kHz for single channel analysis). Current and voltage were monitored simultaneously on a storage oscilloscope (MS-5100A, Iwatsu, Tokyo, Japan) and on a pen recorder (RECTI HORIZ 8K, San-ei, Tokyo, Japan). Traces were stored on a digital video cassette recording system (HV-F93, Mitsubishi, Tokyo, Japan) via a digital audio processor (PCM 501 ESN, Nihon Kohden) for subsequent analysis using the pCLAMP system (Axon Instruments, Foster City, CA, USA). Opening and closing of the channels was detected using a 50% threshold criterion (Colquhoun & Sigworth, 1983). The data are presented as the means ± standard error of the mean (S.E.M.) in the text, and the S.E.M. is indicated by a vertical bar in the figures. A vertical bar was not drawn when the values were smaller than the data point symbol.

Solutions and their application

The ionic composition of the standard external solution was (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) and 10 glucose. In the Mg^{2+}-free standard external solution, $MgCl_2$ was simply removed from the standard external solution. Both external solutions were adjusted to pH 7·4 with tris(hydroxymethyl)aminomethane hydrochloride (Tris-OH) and contained 0·01% bovine serum albumin (fatty acid free). The ionic

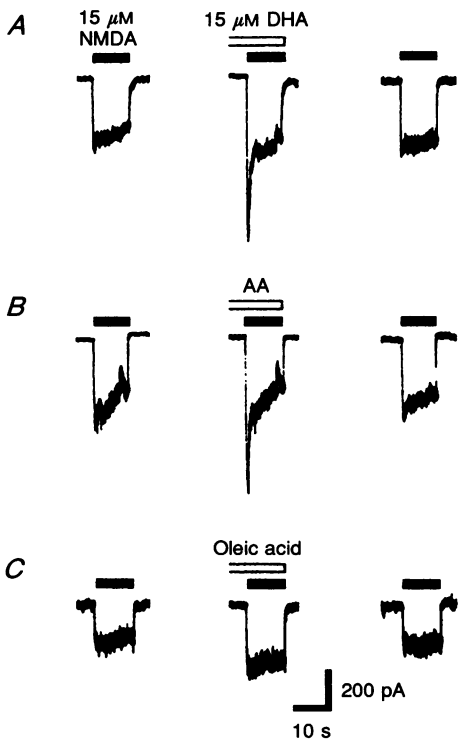


Figure 1. Effects of DHA, AA and oleic acid on NMDA-induced current in Mg^{2+}-free external solution containing 5 μM glycine at a holding potential (V_h) of −60 mV

Potentiation of 15 μM NMDA-induced current by 15 μM DHA (*A*), AA (*B*) and oleic acid (*C*). Horizontal bars indicate the period of application of NMDA and several fatty acids. Neurones were pretreated with fatty acids for at least 20 s before the simultaneous application with NMDA.

composition of the incubation solution was (mM): 125 NaCl, 5 KCl, 1·2 KH_2PO_4, 1·3 $MgSO_4$, 2·4 $CaCl_2$, 24 $NaHCO_3$ and 10 glucose. The solution was adjusted to pH 7·2 with 95 % O_2–5 % CO_2 gas. The ionic composition of the internal (pipette) solution was (mM): 120 CsCl, 20 NaCl, 0·246 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 10 Hepes and 5 Na_2ATP. The pH was adjusted to 7·2 with Tris-OH. Drugs were applied by a rapid application method termed the 'Y-tube' method, as described elsewhere (Murase, Randic, Shirasaki, Nakagawa & Akaike, 1990; Akaike & Shirasaki, 1991). Using this technique, the solution surrounding a dissociated neurone could be completely exchanged within 10–20 ms.

Drugs

The drugs used in the present experiments were arachidonic acid (AA), NMDA, kainic acid, indomethacin, 4-bromophenacylbromide (4-BPB), glycine, thermolysin and bovine serum albumin (fatty acid free) (Sigma, USA), NDGA and staurosporine (Molecular Probes, USA), pronase (Calbiochem, USA), 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7; RBI, USA), and docosapentaenoic acid (DPA), docosatrienoic acid (DTA), oleic acid and palmitic acid (Funakoshi, Tokyo, Japan). DHA was kindly provided by Dr K. Maruyama (Taiyo Fishery Co., Ltd, Japan). DHA and other fatty acids were dissolved in dimethyl sulphoxide (DMSO) at a concentration of between 1 and 30 mM and diluted with a buffer solution sonicated under nitrogen so that the final DMSO concentration was < 0·1 %. All drugs were dissolved in external solution just before use.

RESULTS

In a Mg^{2+}-free external solution containing 5–10 μM glycine, the NMDA-induced inward current could be fully activated in the rat spinal cord dorsal horn and nucleus tractus solitarii neurones (Shirasaki *et al.* 1990). In the present experiment, therefore, NMDA was applied to the cortical neurones immersed in Mg^{2+}-free external solution containing 5 μM glycine. The NMDA-induced inward current in the cortical pyramidal neurones consisted of an initial peak and a successive plateau component at a holding potential (V_h) of −60 mV. Pretreatment with DHA, AA or other fatty acids such as docosapentaenoic acid (DPA), docosatrienoic acid (DTA), oleic acid and palmitic acid did not induce any current. However, successive simultaneous application of DHA, AA, DPA or oleic acid with NMDA increased the 15 μM NMDA-induced current. In particular, both DHA and AA substantially potentiated the peak current component of the NMDA-induced response, whereas they only slightly augmented the steady-state current component. In contrast, oleic acid produced little increase in either component (Figs 1 and 2*B*). Figure 2*A* shows that DHA or AA enhanced the NMDA-induced response in a concentration-dependent manner. The concentration ranges of DHA or AA that potentiated the response to NMDA were between 5 and 30 μM. Both

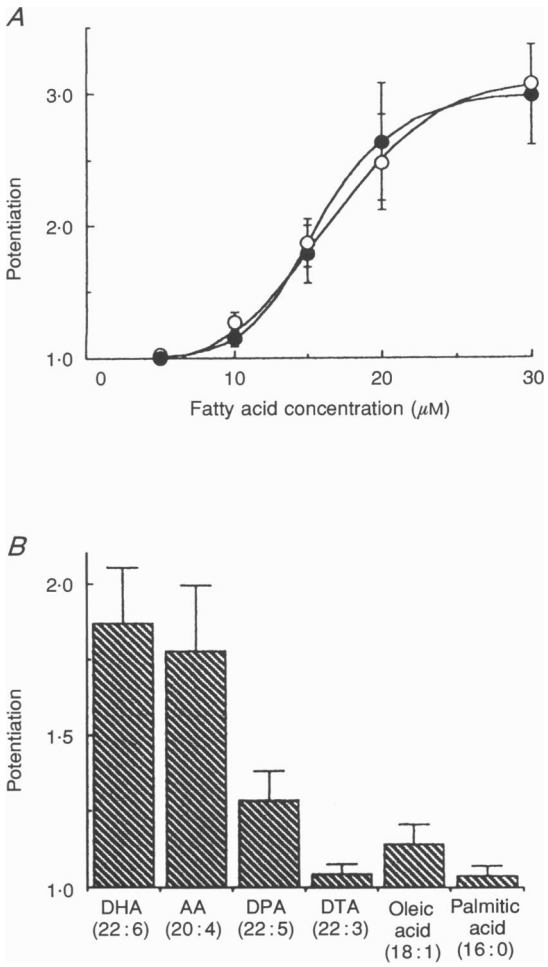


Figure 2. Effects of fatty acids on NMDA-induced current at a V_h of −60 mV

A, facilitatory effects of DHA (○) and AA (●) at various concentrations on 15 μM NMDA-induced response. All currents were normalized to the peak current amplitude induced by 15 μM NMDA alone. Each point represents the mean of ten to twelve neurones. Vertical bars indicate ± S.E.M. *B*, potentiation ratio of 15 μM DHA, AA, docosapentaenoic acid (DPA), docosatrienoic acid (DTA), oleic acid and palmitic acid on 15 μM NMDA-induced current. Each point represents the mean of ten to twelve neurones. Vertical bars indicate + S.E.M.

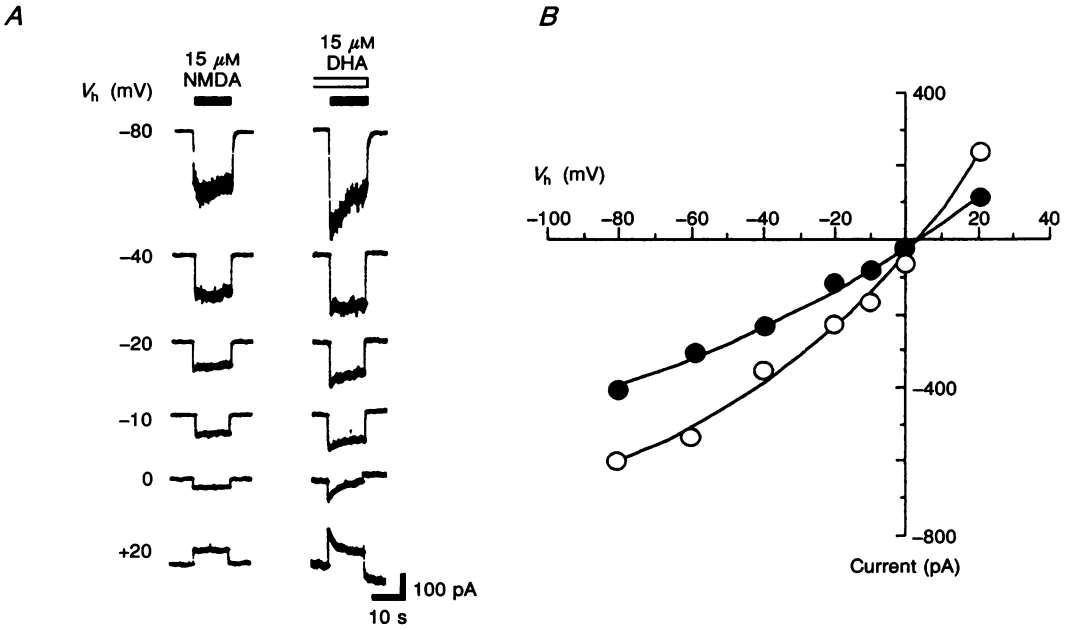
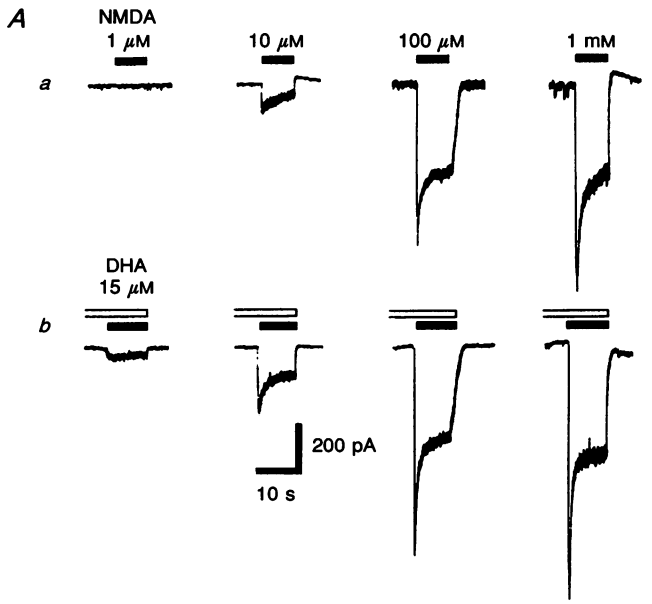


Figure 3. NMDA-induced currents and the current–voltage (I–V) relationship with or without 15 μM DHA

A, 15 μM NMDA-induced currents with or without 15 μM DHA at various holding potentials. The data were all obtained from the same neurone. Horizontal bars indicate the period of NMDA and DHA application. *B*, I–V relationships for the peak NMDA-induced currents with or without DHA. The data were obtained from the same neurone as in *A*.



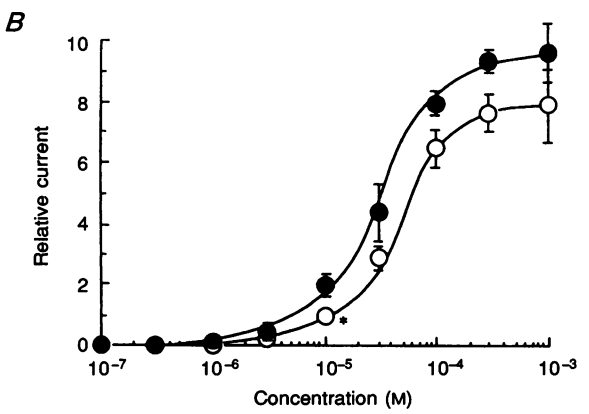


Figure 4. Effects of DHA on the NMDA concentration–response relationship

A, the inward currents induced by NMDA of various concentrations (*a*) and NMDA plus 15 μM DHA (*b*). Horizontal bars indicate the application periods of NMDA and DHA. *B*, potentiation effect of DHA on peak NMDA-induced curent at various concentrations. ○, NMDA alone; ●, NMDA with 15 μM DHA. All currents were normalized to the peak current amplitude induced by 10 μM NMDA alone (*). Each point represents the mean of eight to ten neurones. Vertical bars indicate ± S.E.M.

substances had almost the same potency. The effects of other fatty acids on the NMDA-induced current are also summarized in Fig. 2*B*. DPA, containing one double bond less than DHA, caused a slight but significant potentiation of the NMDA response. Although DPA has a longer chain and more unsaturation than AA, the potentiation ratio was relatively low. Oleic acid, which is a typical mono-unsaturated fatty acid, caused a slight potentiation. DTA, which contains two double bonds less than DHA, and palmitic acid, which is a saturated fatty acid, had no effect on the NMDA-induced response. These results indicate that DHA and AA have the most facilitatory effects on the NMDA-induced response among the fatty acids in mammalian brain.

The voltage dependence of the peak current component induced by 15 μM NMDA was examined in the presence or absence of 15 μM DHA at various holding potentials (Fig. 3). In the current–voltage (I–V) relationship for the NMDA-induced response in the presence of DHA, the DHA potentiated the response to NMDA by 180% at every holding potential, indicating that potentiation of the NMDA-induced response with DHA is not voltage dependent. When the reversal potential of the NMDA-induced current (E_{NMDA}) was estimated from the intercept with the voltage axis of the I–V relationship, the E_{NMDA} was $3{\cdot}82 \pm 0{\cdot}21$ mV ($n = 5$) and $3{\cdot}77 \pm 0{\cdot}36$ mV ($n = 5$) with and without DHA, respectively. Thus, DHA did not alter the reversal potential of the NMDA-induced response.

The augmentative effect of 15 μM DHA on the NMDA-induced responses at the various concentrations was investigated quantitatively. Figure 4*A* shows that DHA caused a 120–200% potentiation of the NMDA-induced response (peak component) in the concentration range between 1 μM and 1 mM. Figure 4*B* shows the concentration–response curve for the peak NMDA-induced current in the absence and presence of DHA at a V_h of −60 mV. Interestingly, although 1 μM NMDA alone did not induce a detectable response, the addition of DHA to 1 μM NMDA induced a significant inward current. The facilitatory effect of DHA was observed even with 1 mM NMDA, which saturates the NMDA receptor site in the presence of 5–10 μM glycine. Moreover, the presence of 5 μM Zn^{2+} or 100 μM spermine did not affect the potentiation by DHA (data not shown). Therefore, these results indicate that DHA acts independently of the glutamate-, glycine-, Zn^{2+}- and polyamine-binding sites on the NMDA receptor.

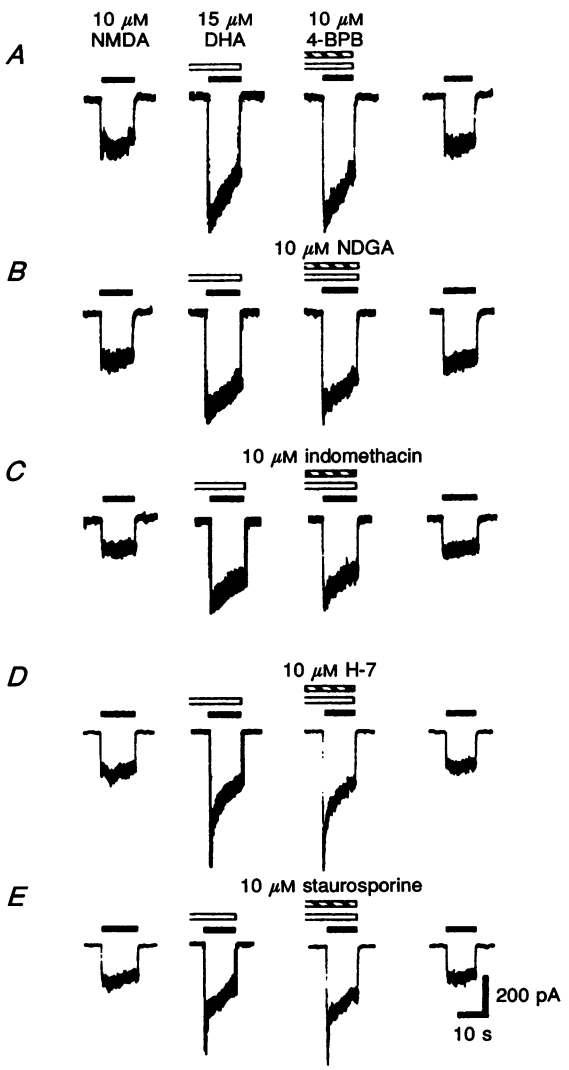


Figure 5. Pharmacological analysis of the potentiation of 10 μM NMDA-induced current by 15 μM DHA
The currents were recorded from different neurones with each blocker. Horizontal bars indicate the application period of NMDA, DHA and blockers. V_h was −60 mV. Recordings *A*–*E* were obtained from different neurones.

Arachidonic acid is a substrate for the production of many biologically active compounds, such as prostaglandins, hydroxyeicosatetraenoic acids and leukotrienes. Similarly, it has become apparent that DHA is a substrate for the production of hydroxydocosahexaenoic acids in the brain (Kim, Karanian, Shingu & Salem, 1990). For this reason, the effects of several AA cascade inhibitors were investigated. 4-BPB (a phospholipase A_2 inhibitor), indomethacin (a cyclo-oxygenase inhibitor) and NDGA (a lipoxygenase inhibitor) did not affect the facilitatory effect of DHA on the NMDA-induced response, ruling out the possibility that the facilitation depends on either the DHA metabolites or AA which is released from the cellular membrane after DHA stimulation (Fig. 5*A–C*).

It is well known that several *cis*-unsaturated fatty acids activate protein kinase C (PKC). In particular, the simultaneous action of oleic acid, AA or DHA with diacylglycerol (DG) dramatically activates PKC (Shinomura, Asaoka, Oka, Yoshida & Nishizuka, 1991). To clarify the involvement of PKC, we examined the effects of H-7 and staurosporine, which are PKC inhibitors, on the potentiation of the NMDA-induced response in the presence of DHA. Neither H-7 nor staurosporine prevented the potentiation of the NMDA-induced response by DHA, indicating that the activation of PKC is not involved in this effect (Fig. 5*D* and *E*). These results suggest that DHA is involved directly in the potentiation mechanism of the NMDA-induced response in the pyramidal neurones of rat cerebral cortex.

The underlying mechanisms of the DHA-induced potentiation of the response to NMDA were examined with the outside-out configuration. To obtain single channel and multichannel current recordings, the inner diameter of the recording pipette was varied (0·6 μm for the single channel current recordings and 1·2 μm for the multichannel ones). As described in the Methods, data were low-pass filtered at 5 kHz and digitized at 2 kHz. Therefore, the open and closed events for longer than 500 μs were defined reliably. Figure 6*A* shows a representative example of the NMDA-operated single channel current recording with the smaller pipette in the absence (upper trace) and presence (lower trace) of 15 μM DHA . The downward deflection shows the channel opening. NMDA-operated channels showed multiple conductances, as previously described (Cull-Candy & Usowicz, 1987). The large conductance of 42 pS was much more frequent than the small conductances of 31, 26 and 18 pS. Similarly, the large conductance of 42 pS was reported in cultured hippocampal neurones (Legendre & Westbrook, 1990) and cerebellar neurones (Cull-Candy & Usowicz, 1989). A short-duration opening of the NMDA channel was observed under the control conditions (1 μM NMDA), while prolongation of open events was frequently induced in the presence of 15 μM DHA. Single channel conductance was constant (42 pS) in the absence and

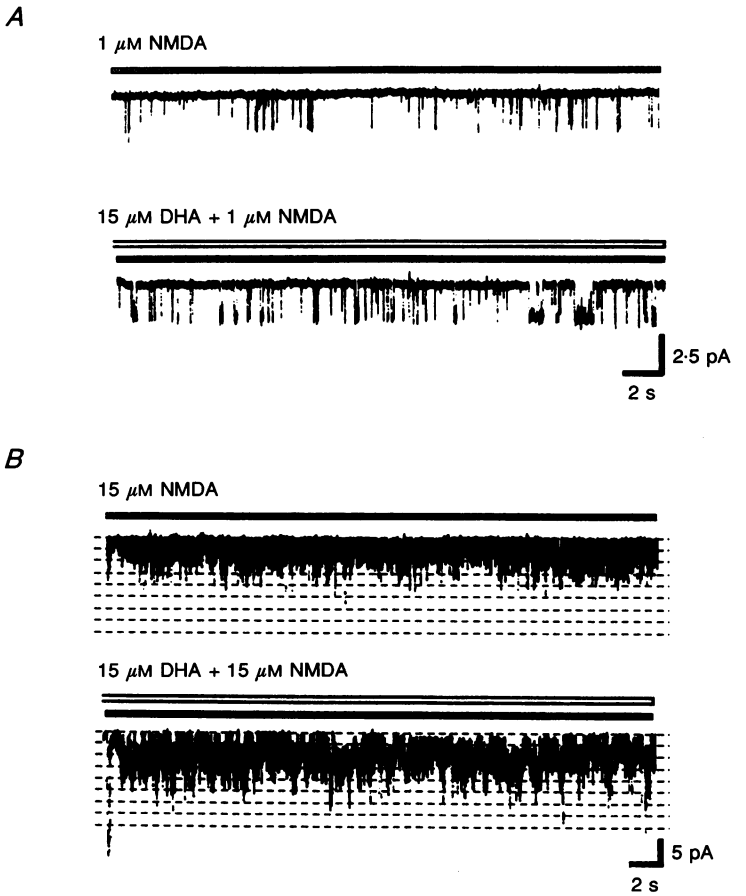


Figure 6. Effect of DHA on NMDA-gated single channel and multichannel currents at a V_h of −60 mV
A, application of 1 μM NMDA with 5 μM glycine to an excised outside-out patch. Addition of 15 μM DHA increased the channel opening frequency of NMDA-gated single channel currents. Horizontal bars indicate the application periods of NMDA and DHA. *B*, application of a high concentration of NMDA (15 μM) led to a maximum of three or four channel openings. The addition of 15 μM DHA caused the opening of more channels. Dashed lines indicate the number of open channels.

presence of DHA at a V_h of −60 mV. The distribution of all open times induced by NMDA was represented by an open-time frequency histogram. Two exponential functions were always required to fit the distribution. Time constants for the fast (τ_{of}) and slow (τ_{os}) components of the open-time histograms were $0{\cdot}21 \pm 0{\cdot}02$ and $9{\cdot}30 \pm 0{\cdot}81$ ms ($n = 11$), respectively. Those of the fast and slow components of opening (τ_{of} and τ_{os}) in the presence of DHA were $0{\cdot}38 \pm 0{\cdot}07$ and $9{\cdot}20 \pm 2{\cdot}26$ ms ($n = 8$), respectively. These time constants were relatively unaffected by 15 μM DHA. The distribution of all closed times induced by NMDA was represented by a closed-time frequency histogram. Two exponential functions were always required to fit the distribution. The time constants for the fast (τ_{cf}) and slow (τ_{cs}) components of the closed-time histograms were $0{\cdot}28 \pm 0{\cdot}09$ and $30{\cdot}84 \pm 1{\cdot}16$ ms ($n = 11$), respectively. Those of the fast and slow components of closure (τ_{cf} and τ_{cs}) in the presence of DHA were $0{\cdot}32 \pm 0{\cdot}09$ and $12{\cdot}50 \pm 1{\cdot}74$ ms ($n = 8$), respectively. DHA decreased the slow component of the closed time (τ_{cs}) without changing the τ_{cf}. Figure 7 shows typical distributions of the open and closed times of channels activated by 1 μM NMDA in the absence and presence of 15 μM DHA. Although the time constants of τ_{of}, τ_{os} and τ_{cf} were not affected by the presence of DHA, τ_{cs} decreased from 31·20 to 11·20 ms. The mean open and closed times for NMDA application were 0·50 and 5·08 ms, respectively in the absence of DHA, and were 0·55 and 0·30 ms, respectively in the presence of DHA. The mean closed time determined from the single NMDA channels activated by DHA was reduced, although the mean open time increased slightly, resulting in a increase in the probability of channel opening. The change in closed time was accompanied by an increase in the number of open events from 242 to 845, and consequently the open probability (P_o) also increased from 3·2 % under the control conditions to 12·9 % in the presence of DHA. When the larger pipette was used, the patch membrane contained a number of channels and the number of open channels was countable. We therefore designated it a multichannel current recording. Figure 6*B* shows an example of the multichannel recording of the NMDA receptor-operated channel. In the absence of DHA (upper trace), five NMDA channels were activated by 15 μM NMDA. When the external solution containing NMDA was replaced by one

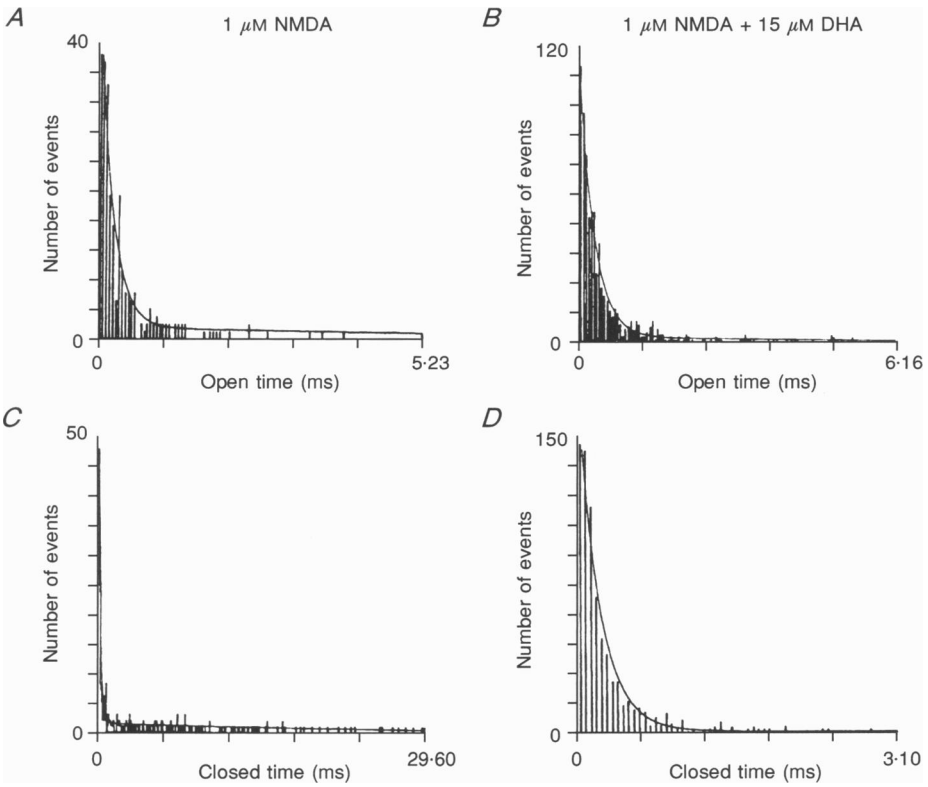


Figure 7. Effect of DHA on the distribution of open and closed times for NMDA-activated channels

A and *B*, distribution of open times of channels activated by 1 μM NMDA with (*B*) or without 15 μM DHA (*A*). Both histograms were fitted with two exponentials. The time constants were estimated as: 0·206 and 10·60 ms in NMDA (*A*); 0·280 and 12·85 ms in NMDA plus DHA (*B*). *C* and *D*, distribution of closed times of channels activated by 1 μM NMDA with (*D*) or without 15 μM DHA (*C*). Both histograms were fitted with two exponentials. The time constants were estimated as: 0·201 and 31·20 ms in NMDA (*C*); 0·20 and 11·20 ms in NMDA plus DHA (*D*).

containing both NMDA and 15 μM DHA, the number of open channels increased. A remarkable increase in the number of activated channels was observed at the start of perfusion. This may have resulted in a potentiation of the peak phase of the NMDA-induced response which was greater than that of the desensitization phase.

The effects of fatty acids on the kainate-induced current were also examined. Application of kainate induced an inward current at a V_h of -60 mV. After pretreatment with DHA for 20 s, the simultaneous application of 20 μM DHA with 100 μM kainate decreased the kainate-induced response. A representative trace of the kainate-induced current that was reduced in the presence of DHA is shown in Fig. 8*A*. DHA reduced the kainate-induced response at all membrane potentials between -80 and $+20$ mV, indicating that the DHA inhibition has no voltage dependence (Fig. 8*B*). DHA did not alter the reversal potential of the kainate-induced response. The inhibitory effect of DHA on the kainate-induced response was concentration dependent (Fig. 8*C*). The effects of several fatty acids (20 μM) on the kainate-induced response are summarized in Fig. 8*D*. Both DHA and AA inhibited the kainate-induced response by about 30 %, whereas oleic acid and palmitic acid had little effect on the response. The rank order of the inhibitory potency was parallel to that of the potentiating NMDA-induced responses. The fact that the modulatory effect of DHA was either potentiation or suppression, depending on the agonists of the glutamate receptors, supports the notion that DHA exerts its effect not through non-specific actions on membrane proteins, but by way of a specifically targeted interaction with a given receptor molecule. Since the modulatory effect of DHA on the kainate-induced response was less potent than that on NMDA-induced response, the former action was not studied any further.

DISCUSSION

Mechanism of enhancement of the NMDA response

Enhancement of whole-cell current can arise from various changes in single channel properties, e.g. increase in mean single channel current amplitude, increase in mean channel open time or increase in the frequency of channel opening. The present single channel experiments showed that DHA increased the channel opening frequency and created shorter states of closure than the control without changing current amplitude. These results taken together indicate

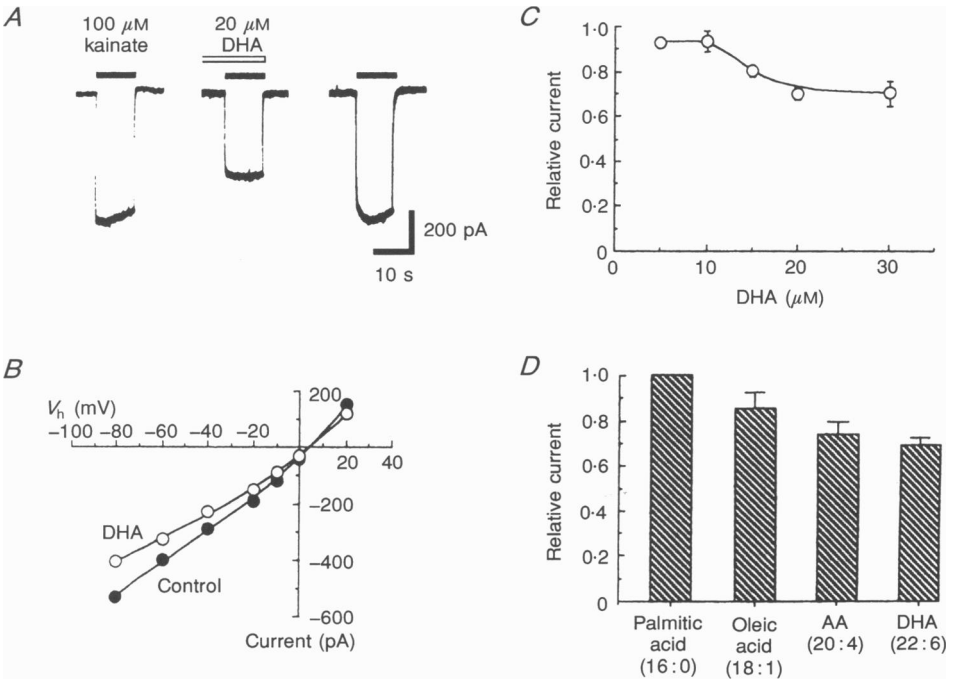


Figure 8. Effect of DHA on kainate-induced current at a V_h of -60 mV
A, inhibition of 100 μM kainate-induced current by 20 μM DHA. Horizontal bars indicate the application period of kainate and DHA. *B*, current–voltage (I–V) relationship of the peak kainate-induced currents at various holding potentials with or without DHA. *C*, inhibitory effect of DHA at various concentrations on 100 μM kainate response. All currents were normalized to the maximum current amplitude induced by 100 μM kainate alone. Each point represents the mean of eight to twelve neurones. Vertical bars indicate $\pm$ S.E.M. *D*, inhibitory effect of 20 μM DHA, AA, oleic acid or palmitic acid on 100 μM kainate-induced response. Each point represents the mean of seven to eight neurones. Vertical bars indicate + S.E.M.

that DHA modifies the NMDA receptor–channel activity, resulting in a P_o increase that manifested itself in a decrease in the time constant of the slow component of closed times. The potentiating effect of DHA was observed even with 1 mM NMDA, which saturates the NMDA receptor site, or in the presence of 5–10 μM glycine, which saturates the glycine-binding site. This finding may indicate that the DHA-binding site that modulates the NMDA response is independent of the glutamate- and glycine-binding sites. The presence of 5 μM Zn^{2+} or 100 μM spermine did not affect the potentiation by DHA, suggesting that DHA acts independently of the Zn^{2+}- and polyamine-binding sites on the NMDA receptor. Moreover, this effect was not inhibited by lipoxygenase and cyclo-oxygenase inhibitors, suggesting that this effect is mediated by free fatty acid itself rather than its metabolites. These results are consistent with the mechanism of AA enhancement of the response to NMDA (Miller *et al.* 1992) and suggest that free DHA may act by binding directly to a novel site on the NMDA receptor or by altering the lipid environment of the NMDA receptor and thereby potentiating the NMDA-induced response. However, there appear to be some differences between types of mammalian central neurones in the facilitatory effect of DHA or AA on the NMDA-induced response. In cerebellar granule cells, the potentiation of NMDA receptor currents was seen at low micromolar levels of AA and was nearly saturated at 10 μM AA with a twofold increase in peak current (Miller *et al.* 1992). In contrast, in the present study, the concentration ranges of DHA or AA that potentiated the NMDA-induced response were between 5 and 30 μM in the cortical pyramidal neurones and, finally, the potentiation ratio reached 3 times. Therefore, there may be some regional differences in the facilitatory effect of DHA or AA on the NMDA-induced response.

Effects of several fatty acids

The major components of fatty acids in mammalian brain are palmitic acid, oleic acid, AA and DHA. Most of the fatty acids are condensed in phospholipids of the cellular membrane (Crawford *et al.* 1976). In particular, DHA and AA are specifically localized in the 2-position of phospholipids, and they are liberated by cellular phospholipases. Liberation of these fatty acids from membrane phospholipids occurs mainly by two pathways. One is the direct liberation through phospholipase A_2. The other is by way of cellular phospholipase C, which cleaves the phospholipids at the phosphate ester bond producing 1,2-DG and, successively, 1,2-DG is broken down by diacylglycerol and monoacylglycerol lipase to yield free fatty acids (Piomelli & Greengard, 1990). After liberation, these fatty acids can diffuse out of the cell and can then play an important role in the potentiation of the NMDA response. In our experiment, oleic acid and palmitic acid scarcely potentiated the NMDA response, but DHA and AA considerably potentiated the response. Moreover, DHA exhibited the most facilitatory effect on the NMDA response in pyramidal neurones of rat cerebral cortex among the fatty acids which have a constant chain length (DHA > DPA > DTA). Therefore, it seems conceivable that the potentiation of the NMDA response is related to an increase in chain length and unsaturation.

What is the role of DHA in the CNS?

It is well known that activation of the NMDA receptor is an absolute requirement for the induction of LTP in the hippocampal CA1 region and neocortex (Collingridge, Kehl & McLennan, 1983; Harris, Ganong & Cotman, 1984; Artola & Singer, 1987). LTP is generally believed to consist of two phases, termed induction and maintenance. The induction of LTP is initiated by the activation of the NMDA receptor, successive postsynaptic entry of Ca^{2+}, and consequent activation of Ca^{2+}-dependent calmodulin and protein kinases (Malenka *et al.* 1989). As the NMDA receptor–channels are blocked by Mg^{2+} at the normal membrane potential, the opening of NMDA receptor–channels requires presynaptic stimulation to release the agonist glutamate and successive postsynaptic depolarization through the non-NMDA receptors to remove the Mg^{2+} blockade. The non-NMDA receptor agonist, quisqualate, not only triggers the depolarization but also activates the release of AA in striatal neurones (Dumuis, Pin, Oomagari, Sebben & Bockaert, 1990). The mechanism of this release is probably through the activation of phospholipase A_2 after the agonists stimulate both ionotropic and metabotropic glutamate receptors. Thus, if DHA and AA released by quisqualate receptor activation diffuse out of the neurones, then the NMDA receptor response can be potentiated by both DHA and AA. Consequently, it might be expected that DHA and AA enhance the induction of LTP. Actually, it has recently been shown that the inhibition of phospholipase A_2 to block AA release prevents the induction of LTP in the hippocampal CA1 region (Okada, Yamagishi & Sugiyama, 1989). Moreover, Williams, Errington, Lynch & Bliss (1989) found that, by combining AA perfusion with a weak stimulation of the perforant path, a long-term enhancement in synaptic strength occurred.

Meanwhile, the activation of the NMDA receptor produces AA release from the postsynaptic neurone and AA acts on the presynaptic terminal to trigger actions, such as the increase in glutamate release (Dumuis, Sebben, Haynes, Pin & Bockaert, 1988). This result suggests a positive feedback among AA, DHA and NMDA receptors, which could underlie the induction phase of LTP. As for the source of the released DHA and AA, it has recently been shown that astrocytes can produce DHA and AA and release these fatty acids into the extracellular fluid (Moore, Toder, Murphy, Dutton & Spector, 1991). DHA and AA,

which potentiate the NMDA response, may diffuse out from neighbouring astrocytes as well as from postsynaptic neurones.

The effect of dietary DHA on the learning ability of rats was reported by Fujimoto *et al.* (1989). In their experiments, the performance of DHA-fed rats in a maze discrimination test was superior to that of rats fed with safflower oil (deficient in DHA), implying that DHA may be involved in specific functions such as learning and memory. The present study suggests a possible candidate for the mechanism of this behavioural effect of DHA. Therefore, DHA released from postsynaptic neurones or astrocytes could potentiate the NMDA-induced response. Consequently, DHA may have a role in promoting the induction of LTP.

REFERENCES

Akaike, N. & Shirasaki, T. (1991). Intracellular ATP regulates GABA responses in rat dissociated hippocampal neurons. In *Role of Adenosine and Adenine Nucleotides in Biological Systems*, ed. Imai, S. & Nakazawa, M., pp. 645–652. Elsevier Science Publishers BV, Amsterdam.

Artola, A. & Singer, W. (1987). Long-term potentiation and NMDA receptors in rat visual cortex. *Nature* **330**, 649–652.

Bliss, T. V. P., Douglas, R. M., Errington, M. L. & Lynch, M. A. (1986). Correlation between long-term potentiation and release of endogenous amino acids from dentate gyrus of anaesthetized rats. *Journal of Physiology* **377**, 391–408.

Collingridge, G. L., Kehl, S. J. & McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral–commissural pathway of the rat hippocampus. *Journal of Physiology* **334**, 33–46.

Colquhoun, D. & Sigworth, F. J. (1983). Fitting and statistical analysis of single-channel current records. In *Single-channel Recording*, ed. Sakmann, B. & Neher, E., pp. 191–264. Plenum Press, New York and London.

Crawford, M. A., Casperd, N. M. & Sinclair, A. J. (1976). The long chain metabolites of linoleic and linolenic acids in liver and brain in herbivores and carnivores. *Comparative Biochemistry and Physiology* **54B**, 395–401.

Cull-Candy, S. G. & Usowicz, M. M. (1987). Multiple-conductance channels activated by excitatory amino acids in cerebellar neurones. *Nature* **325**, 525–528.

Cull-Candy, S. G. & Usowicz, M. M. (1989). On the multiple-conductance single channels activated by excitatory amino acids in large cerebellar neurones of the rat. *Journal of Physiology* **415**, 552–582.

Dhopeshwarkar, G. A. & Subramanian, C. (1975). Metabolism of linolenic acid in developing brain: I. Incorporation of radioactivity from 1-^{14}C linolenic acid into brain fatty acids. *Lipids* **10**, 238–241.

Dumuis, A., Pin, J. P., Oomagari, K., Sebben, M. & Bockaert, J. (1990). Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. *Nature* **347**, 182–184.

Dumuis, A., Sebben, M., Haynes, L., Pin, J. P. & Bockaert, J. (1988). NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature* **336**, 68–70.

Fujimoto, K., Yao, K., Miyazawa, T., Hirano, H., Nishikawa, M., Kimura, S., Maruyama, K. & Nonaka, M. (1989). The effect of dietary docosahexaenoate on the learning ability of rats, In *Health Effects of Fish and Fish Oils*, ed. Chandra, R. K., pp. 275–284. ARTS Biomedical Publishers & Distributors, Newfoundland.

Hadjiagapiou, C. & Spector, A. A. (1987). Docosahexaenoic acid metabolism and effect on prostacyclin production in endothelial cells. *Archives of Biochemistry and Biophysics* **253**, 1–12.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Harris, E. W., Ganong, A. H. & Cotman, C. W. (1984). Long-term potentiation in the hippocampus involves activation of *N*-methyl-D-aspartate receptors. *Brain Research* **323**, 132–137.

Ito, C., Wakamori, M. & Akaike, N. (1991). Dual effect of glycine on isolated rat suprachiasmatic neurons. *American Journal of Physiology* **260**, C213–218.

Kauer, J. A., Malenka, R. C. & Nicoll, R. A. (1988). NMDA application potentiates synaptic transmission in the hippocampus. *Nature* **334**, 250–252.

Kim, H. Y., Karanian, J. W., Shingu, T. & Salem, J. N. (1990). Sterochemical analysis of hydroxylated docosahexaenoates produced by human platelets and rat brain homogenate. *Prostaglandins* **40**, 473–490.

Legendre, P. & Westbrook, G. L. (1990). The inhibition of single *N*-methyl-D-aspartate-activated channels by zinc ions on cultured rat neurones. *Journal of Physiology* **429**, 429–449.

Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A. & Waxham, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature* **340**, 554–557.

Martinez, M. (1990). Severe deficiency of docosahexaenoic acid in peroxisomal disorders: a defect of delta 4 desaturation? *Neurology* **40**, 1292–1298.

Miller, B., Sarantis, M., Traynelis, S. F. & Attwell, D. (1992). Potentiation of NMDA receptor currents by arachidonic acid. *Nature* **355**, 722–725.

Moore, S. A., Toder, E., Murphy, S., Dutton, G. R. & Spector, A. A. (1991). Astrocytes, not neurons, produce docosahexaenoic acid (22:6–3) and arachidonic acid (20:4–6). *Journal of Neurochemistry* **56**, 518–524.

Murase, K., Randic, M., Shirasaki, T., Nakagawa, T. & Akaike, N. (1990). Serotonin suppresses *N*-methyl-D-aspartate responses in acutely isolated spinal dorsal horn neurons of the rat. *Brain Research* **525**, 84–91.

Neuringer, M., Connor, W. E., Lin, D. S., Barstad, L. & Luck, S. (1986). Biochemical and functional effects of prenatal and postnatal omega-3 fatty acid deficiency on retina and brain in rhesus monkeys. *Proceedings of the National Academy of Sciences of the USA* **83**, 4021–4025.

Okada, D., Yamagishi, S. & Sugiyama, H. (1989). Differential effects of phospholipase inhibitors in long-term potentiation in the rat hippocampal mossy fiber synapses and Schaffer/commissural synapses. *Neuroscience Letters* **100**, 141–146.

Piomelli, D. & Greengard, P. (1990). Lipoxygenase metabolites of arachidonic acid in neuronal transmembrane signalling. *Trends in Pharmacological Sciences* **11**, 367–373.

Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991). Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: its possible implications. *Proceedings of the National Academy of Sciences of the USA* **88**, 5149–5153.

Shirasaki, T., Nakagawa, T., Wakamori, M., Tateishi, N., Fukuda, A., Murase, K. & Akaike, N. (1990). Glycine-insensitive desensitization of *N*-methyl-D-aspartate receptors in acutely isolated mammalian central neurons. *Neuroscience Letters* **108**, 93–98.

Söderberg, M., Edlunk, C., Kristensson, K. & Dallner, G. (1991). Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. *Lipids* **26**, 421–425.

Stubbs, C. D. & Smith, A. D. (1984). The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochimica et Biophysica Acta* **779**, 89–137.

TINOCO, J., BABCOCK, R., HINCENBERG, I., MEDWADOWSKI, B. & MILJANICH, P. (1978). Linolenic acid deficiency: changes in fatty acid patterns in female and male rats raised on a linolenic acid-deficient diet for two generations. *Lipids* **13**, 6–17.

WILLIAMS, J. H. & BLISS, T. V. P. (1988). Induction but not maintenance of calcium-induced long-term potentiation in dentate gyrus and area CA1 of the hippocampal slice is blocked by nordihydroguaiareetic acid. *Neuroscience Letters* **88**, 81–85.

WILLIAMS, J. H., ERRINGTON, M. L., LYNCH, M. A. & BLISS, T. V. (1989). Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus. *Nature* **341**, 739–742.

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