Developmental increase in hippocampal endocannabinoid mobilization: role of metabotropic glutamate receptor subtype 5 and phospholipase C

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1Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan, Republic of China; 2Program in Neuroscience, University of Maryland School of Medicine, Baltimore, Maryland; 3Departments of Physiology and Psychiatry, University of Maryland School of Medicine, Baltimore, Maryland; and 4Department of Pharmacology, University of Maryland School of Medicine, Baltimore, Maryland

Submitted 6 February 2014; accepted in final form 26 August 2014

Liang SL, Alger BE, McCarthy MM. Developmental increase in hippocampal endocannabinoid mobilization: role of metabotropic glutamate receptor subtype 5 and phospholipase C. J Neurophysiol 112: 2605–2615, 2014. First published September 3, 2014; doi:10.1152/jn.00111.2014.—Endocannabinoids (eCBs) released from postsynaptic neurons mediate retrograde suppression of neurotransmitter release at central synapses. eCBs are crucial for establishing proper synaptic connectivity in the developing nervous system. Mobilization of eCBs is driven either by a rise in intracellular Ca2+ (de polarization-induced suppression of inhibition, DSI) or postsynaptic G protein-coupled receptors (GPCRs) that activate phospholipase C beta (PLCβ). To determine whether eCB mobilization changes between neonatal and juvenile ages, we used whole cell voltage-clamp recordings of CA1 neurons from rat hippocampal slices at postnatal days 1–18 (neonatal) and 19–43 (juvenile), because many neurophysiological parameters change dramatically between approximately postnatal days 18–20. We found that DSI was slightly greater in juveniles than in neonates, while eCB mobilization stimulated by GPCRs was unchanged. However, when DSI was elicited during GPCR activation, its increase was much greater in juveniles, suggesting that eCB mobilization caused by the synergy between the Ca2+ and GPCR pathways is developmentally upregulated. Western blotting revealed significant increases in both metabotropic type glutamate receptor 5 (mGluR5) and PLCβ1 proteins in juveniles compared with neonates. Responses to pharmacological activation or inhibition of PLC implied that eCB upregulation is associated with a functional increase in PLC activity. We conclude that synergistic eCB mobilization in hippocampal CA1 neurons is greater in juveniles than in neonates, and that this may result from increases in the mGluR5-PLCβ1 eCB pathway. The data enhance our understanding of the developmental regulation of the eCB system and may provide insight into diseases caused by improper cortical wiring, or the impact of cannabis exposure during development.

DSI; CA1 region; G protein-coupled receptors; mAChRs; whole cell voltage-clamp; Western blot

Unlike conventional neurotransmitters that are released from presynaptic nerve terminals and mediate anterograde neurotransmission in central synapses, endocannabinoids (eCBs) are produced and released from postsynaptic neurons and act via retrograde transmission on presynaptic cannabinoid type I receptors (CB1R) to suppress release of neurotransmitters, e.g., GABA and glutamate (Kano et al. 2009). Mobilization of eCBs can be achieved in three ways. One is via a strong depolarization of postsynaptic neurons which opens voltage-gated calcium (Ca2+) channels and increases intracellular Ca2+ concentration, [Ca2+]i, to micromolar levels (Lenz and Alger 1999) which mobilizes eCBs via a phospholipase C (PLC)-independent pathway (Hashimotodani et al. 2005). Since Ca2+-dependent eCB mobilization can produce either depolarization-induced suppression of inhibition (DSI) (Pitler and Alger 1992) or of excitation (Kreitzer and Regehr 2001), depending on whether excitatory or inhibitory synapses are involved, this purely Ca2+-dependent eCB process can be designated generally as “eCBCa2+”. The second mechanism, requiring activation of postsynaptic G protein-coupled receptors (GPCRs) (Maejima et al. 2005; Varma et al. 2001), is largely independent of Ca2+ and involves a diacylglycerol-lipase-α-dependent (Gao et al. 2010; Tanimura et al. 2010) pathway that may include PLC. This process can be designated generally as “eCBGPCR”. The third mechanism is a synergistic interaction between Ca2+ and the GPCR-activated pathways (Kim et al. 2002; Varma et al. 2001). The increase in [Ca2+]i interacts with the GPCR activation and produces an elevated level of eCB release, a process designated “eCBCa2+-GPCR”.

In the hippocampus, eCB-mediated short-term depression of synaptic GABA release, i.e., DSI (Pitler and Alger 1992), is produced when eCBs are released from pyramidal cells (Ohnoshosaku et al. 2001; Wilson and Nicoll 2001) and suppress inhibitory postsynaptic currents (IPSCs) by activating CB1Rs located mainly on cholecystokinin (CCK)-containing GABAergic inhibitory interneurons (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999). Activation of either the muscarinic acetylcholine receptors (mAChRs; Kim et al. 2002; Ohnoshosaku et al. 2003) or group I metabotropic type glutamate receptors (mGluR1 and mGluR5) (Ohnoshosaku et al. 2002; Varma et al. 2001) enhance DSI, which are examples of eCBCa2+GPCR in adult hippocampus. [Note: Instead of “eCBCa2+”, Kano et al. (2009) use “CaER”, where “ER” is “endocannabinoid release,” instead of “eCBGPCR,” they use “basal RER,” “receptor-activated ER,” and instead of “eCBCa2+-GPCR”, they use “Ca2+-assisted RER.” There is no difference in the phenomena themselves.]

Although modulation of synaptic transmission by eCB signaling is increasingly well understood in adult brain, less is known about eCB system regulation during postnatal development. CB1Rs (Harkany et al. 2007; Wang et al. 2003), eCBs (Berrendero et al. 1999; Fernandez-Ruiz et al. 2000), together with their synthesizing (Berghuis et al. 2007; Watson et al. 2008) and degrading enzymes (Harkany et al. 2007), can all be...
detected from the earliest stages of embryonic development and throughout the pre- and postnatal periods (Basavarajappa et al. 2009; Deshmukh et al. 2007) in various brain areas, including hippocampus. Moreover, CB1R mRNA expression levels increase gradually in the hippocampus from the fetal period to adulthood (Harkany et al. 2007). During embryonic and prenatal development, eCB signaling regulates neuronal migration (Berghuis et al. 2007; Morozov and Freund 2003b), axonal elongation (Gomez et al. 2008; Mulder et al. 2008) and synaptogenesis (Kim and Thayer 2001). Moreover, disruption of eCB signaling during early postnatal development alters cortical activity patterns (Bernard et al. 2005), suggesting a role for eCBs in activity-dependent synaptic refinement (Deshmukh et al. 2007). How the mobilization of eCBs is regulated during postnatal development in hippocampus remains to be elucidated, however.

To investigate the developmental issues related to eCB signaling, we have used whole cell voltage-clamp recordings of electrically (field stimulation) evoked inhibitory postsynaptic currents (eIPSCs) in hippocampal CA1 neurons and Western blot analysis of proteins that are involved in eCB signaling. We focused mainly on DSI and the enhancement of DSI that is 1 vs. 2 MΩ; 80% compensation) was monitored using a stability (H11349) was monitored using a stability (H11349) was monitored using a stability (H11349) was monitored using a stability (H11349).

**Brain slice preparation.** Brains of neonatal and juvenile rats (114 males and 63 females) were rapidly removed following sedation and decapitation, in accordance with standard approved protocols. Horizontal, 400-μm-thick brain slices that included the hippocampus were prepared using a vibratome (Vibratome series 1000, St. Louis, MO) in ice-cold sucrose solution, where NaCl in the artificial cerebral spinal fluid (ACSF) was replaced by an isosmotic concentration of sucrose. The ACSF composition was as follows (in mM): 130 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 10 glucose, 1 MgCl2, 2 CaCl2 (saturated with 95% O2/5% CO2). The slices were incubated at 34°C for 20 min and then allowed to recover for at least 1 h at room temperature in an interface holding chamber filled with humid 95% O2/5% CO2 before the experiment. In most studies, the slices were incubated with ω-agatoxin GVIA (agatoxin, 300 nM), an irreversible P/Q-type Ca2+ channel blocker, for 30–60 min prior to experiments (“pretreatment”) to depress occurrence of eCB-insensitive IPSCs (e.g., Lenz et al. 1998; Wilson et al. 2001) during recordings. Agatoxin reduces the total GABA<sub>A</sub> receptor-mediated IPSC elicited with extracellular stimulation in CA1 by ~60% within ~25 min of treatment in our hands (Lafoucade and Alger 2008). However, it is too expensive to use in routine perfusion and since agatoxin is essentially irreversible within the time frame of the experiments, e.g., Wheeler et al. 1994), we pretreated slices with it. Stimulus intensities required to evoke IPSCs in agatoxin-pretreated slices appear to be higher than in untreated slices, as expected, since a large fraction of the interneurons, especially parvalbumin-expressing interneurons, depend exclusively on activation of agatoxin-sensitive (P/Q-type) Ca2+ channels to release GABA.

**Whole cell voltage-clamp recordings.** Visualized whole cell voltage-clamp recordings from CA1 pyramidal neurons were made in hippocampal slices at room temperature (20–22°C) on a fixed stage upright microscope (Nikon Eclipse E600FN, Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (DAGE-MTI, Michigan City, IN) using a ×60 water immersion objective. Carrying out the experiments at room temperature improves the viability of slices in the visualized preparation and is quite common in experiments like these. Slices were superfused with oxygenated ACSF containing 6,7-dinitrooxidoline-2,3-dione (DNQX; 10 μM) and d-(-)-2-amino-5-phosphonopentanoic acid (AP5; 50 μM) to block ionotropic glutamatergic responses. The ACSF flowed through the chamber at a rate of ~1.5 ml/min. Patch electrodes (borosilicate glass, WPI, Sarasota, FL) had resistances of 4–6 MΩ in the bath when filled with the internal solution, which contained the following (in mM): 90 CsCl, 50 CsCl, 0.2 Cs<sub>2</sub>BAPTA, 10 HEPES, 1 MgCl<sub>2</sub>, 2.5 phosphocreatine-2Na, 2 Mg-ATP, and 0.3 GTP-Tris, 5 lidocaine, N-ethylmaleimide (QX-314) titrated to pH 7.2–7.3 with 3 M CsOH, and then allowed to recover for at least 1 h at room temperature in an interface holding chamber filled with humid 95% O2/5% CO2 before being homogenized in 400 μl of lysis buffer (pH 7.4; 0.8% trizma base, 0.9% NaCl, 1% igepal, 1 M deoxycylic acid, 1% EDTA, 0.1% phosphatase inhibitor and 0.1% protease inhibitor). The sections were centrifuged for 30 min at 3,000 rpm, and the supernatant was collected. The homogenate was then subjected to a Bradford protein assay to determine standardize protein levels in subsequent Western blotting analyses. Ten micrograms of protein from each animal were electrophoresed in separate lanes on an

**MATERIALS AND METHODS**

**Animals.** Sprague-Dawley rats were bred in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facilities of the University of Maryland School of Medicine, USA, or the Chang Gung University, Taiwan, and maintained on a standard light cycle with ad libitum food and water. All procedures were approved by the Institutional Animal Care and Use Committees for both institutions. Pregnant dams were checked each morning, and the day when pups were first found was deemed postnatal day 0 (PN0). Animals were divided into neonatal (PN1–18) and juvenile (PN19–43) groups because a large number of physiological parameters in the hippocampus change dramatically in the range of PN18–20, which is also essentially the time of weaning (PN19–21) in the animal colonies. Parameters changing include the relative abundance of α<sub>1</sub>- vs. α<sub>2</sub>-subunits of the GABA<sub>A</sub>-receptor (Davis et al. 1999) and the changes in depolarizing vs. hyperpolarizing GABA action (Ben-Ari et al. 2012). Juvenile animals are independent but not yet reproductively mature.

**Brain slice preparation.** Brains of neonatal and juvenile rats (114 males and 63 females) were rapidly removed following sedation and decapitation, in accordance with standard approved protocols. Horizontal, 400-μm-thick brain slices that included the hippocampus were prepared using a vibratome (Vibratome series 1000, St. Louis, MO) in

**J Neurophysiol.** doi:10.1152/jn.00111.2014 • www.jn.org
or and P or one-way ANOVA followed by Newman-Keuls test for multiple
in drug(s)/control DSI. Normalized DSI was calculated as {
Values of two or three DSI trials were averaged to obtain a mean DSI
amplitude of five eIPSCs before depolarization pulse.

The integrative gray-scale pixel area density of mGluR5 bands was captured with a charge-coupled device camera and quantified using National Institutes of Health Image software (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). The membranes containing PLCβ1 bands were developed with enhanced chemiluminescent horseradish peroxidase substrate (Bionovas Biotechnology, Ann Arbor, MI) and then exposed to autoradiography film, and band intensity was measured with Image J software. The mGluR5 and PLCβ1 proteins were detected as bands of relative molecular mass of 130 and 150 kDa, respectively. Data were expressed as the ratios of either mGluR5 or PLCβ1 to respective Ponceau S signals.

Data analysis. DSI was calculated as follows: DSI (%) = (1 – mean amplitude of three eIPSCs after depolarization pulse/mean amplitude of five eIPSCs before depolarization pulse) × 100%. Values of two or three DSI trials were averaged to obtain a mean DSI in a given condition. Normalized DSI was calculated as [(DSI tested in drug(s)/control DSI) × 100%]. Data are expressed as means ± SE, and P values are calculated from Student’s unpaired or paired t-tests or one-way ANOVA followed by Newman-Keuls test for multiple comparisons, with significance levels assessed at P < 0.05, P < 0.01 or P < 0.005, with two-tailed tests.

Chemicals sources. DHPC, DNQX, and d-AP5 were purchased from Tocris Cookson (Bristol, UK), α-Agatoxin IVA was obtained from Peptides International (Louisville, KY), U-73122, U-73343 and 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS) were purchased from Calbiochem (Gibbstown, NJ), 2,4,6-Trimethyl-N-(ortho-3-trifluoromethyl-phenyl)-benzenesulfonamide (o-3M3FBS) was ordered from Tocris (Minneapolis, MN). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

Developmental change in eCB_{Ca} in response to a strong depolarization of CA1 neurons. A transient depolarization of hippocampal CA1 neurons causes DSI (Pitler and Alger 1992), which is mediated by eCBs (Wilson and Nicoll 2001), in juvenile and PN1–7 neonatal rats (Bernard et al. 2005). Since synthesis and release of eCBs cannot be distinguished electrophysiologically, we refer to the combination of both processes as “mobilization”. To examine whether eCB mobilization exhibits a developmental change, we first tested DSI by giving cells 1-s-long depolarizing voltage steps every 2 min through the whole cell pipette. However, in the youngest cohort of animals (PN1–9), essentially no DSI could be observed in normal ACSF, and accordingly we used 5-s-long depolarizing voltage steps (as numerous others have done, e.g., Wilson and Nicoll 2001) and recorded eIPSCs that were evoked at 4-s intervals during neonatal and juvenile stages (see MATERIALS AND METHODS). DSI in neonates amounted to an average depression of 21 ± 1% of the eIPSC amplitude (Fig. 1, A and E), which is consistent with published results (Bernard et al. 2005). Since there were no sex differences in the DSI values recorded from neonatal (n = 43 males and n = 29 females) or juvenile (n = 18 for each sex) groups, and there was no sex and age interaction (P value = 0.78, nonsignificant, two-way ANOVA), the male and female results were lumped together within the two age cohorts. DSI in juveniles was significantly
greater than in neonates (a depression of $31 \pm 2\%$, an increase to $147\%$ of the neonate value, $n = 72$ in neonates, $n = 36$ in juveniles, $P < 0.01$; Fig. 1, A, C, and E).

The main receptor for eCBs in the brain, CB1R, is heavily concentrated on CCK-containing GABAergic inhibitory interneurons in hippocampus (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999). Although CCK-expressing cells constitute one of the two major basket cell classes, non-CCK interneurons are present, and our extracellular stimulation will activate them as well. Many of the non-CCK/non-CB1R expressing interneurons release GABA via P/Q-type Ca\(^{2+}\) channels (e.g., Freund and Katona 2007; c.f., Lenz et al. 1998; Wilson et al. 2001), which are blocked by the selective P/Q-type Ca\(^{2+}\) channel blocker, agatoxin, whereas CCK interneurons release GABA exclusively via N-type Ca\(^{2+}\) channels, which are insensitive to agatoxin. Therefore, to reduce co-occurrence of eCB-insensitive IPSCs that could confound the measurement of DSI, slices were pretreated with agatoxin (300 nM) for 30–60 min prior to the experiment (see MATERIALS AND METHODS). Agatoxin is irreversible within the time frame of the experiments (c.f., Wheeler et al. 1994). Following pretreatment with agatoxin, DSI recorded from CA1 neurons of both neonates and juveniles showed significant increases above their respective non-agatoxin treated groups (DSI increased from $21 \pm 1\%$ to $37 \pm 2\%$ in neonates, $n = 80$, $P < 0.01$; and from $31 \pm 2\%$ to $45 \pm 3\%$ in juveniles, $n = 24$, $P < 0.01$; Fig. 1, B, D, and E). This indicates that the difference between neonatal and juvenile responses can mainly be attributed to a relatively smaller contribution of the eCB-sensitive IPSCs to the total neonatal eIPSCs. For these and all further experiments in agatoxin-pretreated slices, we used 1-s depolarizing voltage steps to induce DSI, to avoid any possible “ceiling effect” that might occur with 5-s steps and maximal DSI.

After agatoxin pretreatment, the difference between neonatal and juvenile DSI was smaller but still significant (37% vs. 45%; an increase of 22%; $P = 0.04$; Fig. 1E). The reduction in the difference probably reflects developmental changes in the relative populations of agatoxin-sensitive and -insensitive interneurons (Tricoire et al. 2011). The remaining difference reflects the true developmental change in eCB\(_{\text{Ca}}\). Again, there were no sex differences in the DSI values recorded in neonatal ($n = 45$ for males and $n = 35$ for females) or juvenile ($n = 12$ for each sex) groups, and there was no sex and age interaction ($P = 0.23$, nonsignificant, two-way ANOVA, data not shown). The male and female results were lumped together within the two age cohorts in this and all subsequent experiments.

Developmental increase in eCB\(_{\text{Ca}}\)GPRC initiated by a mGluR agonist. Activation of mACHRs induces the mobilization of eCBs in young adult hippocampal CA1 neurons (Kim et al. 2002). Muscarinic AChRs are present in the hippocampus of neonatal rats (Guo-Ross et al. 2007). We found that, in the absence of a DSI step, application of carbachol (CCh, 5 \mu M) alone had almost no effect on the eIPSCs in either neonates or juveniles ($10 \pm 7\%$, $n = 7$ in neonates; $9 \pm 2\%$, $n = 5$, in juveniles; $P = 0.86$). This did not reflect an absence of CB1Rs, because the recorded cells were capable of producing robust DSI before the CCh application (data not shown). Moreover, the results did not differ between neonates and juveniles. Hence, there was no detectable developmental change in eCB\(_{\text{GPRC}}\) in hippocampal CA1 neurons, although we cannot rule out the occurrence of subtle changes below our level of resolution.

In agatoxin-pretreated slices, DSI produced by a 1-s depolarization caused an average eIPSC depression of $17 \pm 3\%$ in neonates (Fig. 2, A and C; $n = 6$), and application of CCh for 5 min significantly increased DSI (mean DSI was 29$\% \pm 6\%$; $P < 0.03$, paired t-test, Fig. 2, A and C). On the other hand, DSI recorded from juveniles in this group was on average $10 \pm 2\%$ in control ACSF, but, following application of CCh, was increased to $57 \pm 7\%$ (Fig. 2, B and D; $n = 6$, $P < 0.001$, paired t-test). The fact that CCh increased DSI in juveniles to a greater extent than in neonates (Fig. 2E; $n = 6$, $P < 0.01$, unpaired t-test) suggests there is a developmental increase in the combined GPCR plus Ca\(^{2+}\) mechanism, i.e., in eCB\(_{\text{Ca}}\)GPRC (e.g., Varma et al. 2001; see Kano et al. 2009 for review). Note that, in addition to increasing the peak magnitude of DSI, the eCB\(_{\text{Ca}}\)GPRC process also prolongs its duration, undoubtedly reflecting the more complex kinetics associated with the biochemical pathway stimulated by the GPCR compared with that of the Ca\(^{2+}\) transient alone, which largely determines the duration of DSI.

Developmental increase in eCB\(_{\text{Ca}}\)GPRC initiated by group I mGluR agonist. DSI is also increased in adult animals by the selective group I mGluR agonist, DHPG (Ohno-Shosaku et al. 2002; Varma et al. 2001). To determine whether the developmental difference in eCB\(_{\text{Ca}}\)GPRC occurs only when mAChRs are stimulated, or is also found when other GPCRs, in particular group I mGluRs (mGluR1 and mGluR5) are stimulated, we recorded DSI before and after application of the selective group I mGluR agonist, DHPG (1 \mu M), in agatoxin-pretreated slices. DSI in neonates was increased by a 5-min application of DHPG (from $22 \pm 2\%$ to $31 \pm 4\%$ in DHPG; Fig. 3, A and C; $n = 24$, $P < 0.01$, paired t-test). In comparison, juvenile DSI increased on average from $20 \pm 5\%$ to $46 \pm 4\%$ by DHPG (Fig. 3, B and D; $n = 11$, $P < 0.001$, paired t-test). The increase in DSI caused by DHPG was much greater in juveniles than in neonates (Fig. 3E; $P < 0.01$, unpaired t-test). The results with DHPG are consistent with those with CCh and suggest that a common mechanism is involved in the developmental increase in eCB\(_{\text{Ca}}\)GPRC.

Developmental increases in both mGluR5 and PLC\(_{\beta 1}\) protein expression in hippocampus. Because the results obtained with both mAChR and mGluR agonists were essentially the same, we chose to focus on the group I mGluRs. mGluR5 is abundantly expressed in hippocampal CA1 neurons (Shigemoto et al. 1993) and may be involved in low-frequency, stimulation-enhanced DSI in neonates (Zhu and Lovinger 2010). PLC\(_{\beta 1}\) is proposed to mediate eCB\(_{\text{GPRC}}\) and to be the coincidence detector for eCB\(_{\text{Ca}}\)GPRC in hippocampal neurons (Hashimotodani et al. 2005). If the developmental increase in DSI caused by DHPG is related to changes in the eCB\(_{\text{Ca}}\)GPRC pathway, we predicted that there might be developmental increases in the protein expression of mGluR5, PLC\(_{\beta 1}\), or both. To test this prediction, we saved the recorded hippocampal slices and carried out Western blot analyses on them (see MATERIALS AND METHODS). We observed increases in protein expression levels in juveniles over the neonatal values for both mGluR5 (an increase of $58\%$; $n = 10$ for neonates, $n = 25$ for juveniles, $P < 0.05$) and PLC\(_{\beta 1}\) (an increase of $89\%$; $n = 9$ for both neonates and juveniles, $P < 0.001$; see Fig. 4, A and B). These results suggest that the developmental increase in
eCB$_{Ca+GPCR}$ may be accounted for by increases in these components of the GPCR pathway.

Inhibitor of PLC activity reduces eCB$_{Ca+GPCR}$ in both juveniles and neonates. If the eCB$_{Ca+GPCR}$ process is less effective in neonates, as our data suggest, perhaps because of the differences in mGluR5 or PLC, then altering the efficacy of the pathway should have differential effects in neonates and juveniles. To test this prediction, we used pharmacological agents to affect the activity of PLC in agatoxin-pretreated slices. We began by testing the PLC inhibitor U-73122 (5 μM), and its inactive analog, U-73343 (5 μM). Neither of these agents significantly affected DSI in control conditions in neonates or juveniles (18 ± 3% to 22 ± 3%, n = 7, paired t-test, P = 0.07 in neonates and 19 ± 2% to 20 ± 3%, n = 12, paired t-test, P = 0.65, in juveniles). Neither agent affected DSI in neonates or juveniles by 30 μM 3M3FBS (Fig. 6, I). The active compound, n-Methyl-d-aspartate (NMDA) receptor agonist, significantly increased DSI in cells in both neonates (16 ± 3% to 27 ± 3%, n = 15, P < 0.001; Fig. 5A), or in juveniles (n = 10, P < 0.001; Fig. 5C), and, as before, DHPG caused a greater increase in DSI in the juveniles (n = 10) than in the neonatal (n = 15) animals (P < 0.05, unpaired t-test). However, the active PLC inhibitor, U-73122 prevented the enhancement of DSI by DHPG in both neonates (n = 10, P = 0.9; Fig. 5B), and in juveniles (n = 8; P = 0.06, paired t-test, Fig. 5D). Summary data are shown in Fig. 5E.

Activator of PLC activity induces eCB$_{Ca+GPCR}$ in neonates but not juveniles. Although the PLC antagonist blocked the increase in DSI caused by DHPG in both groups, the difference in the initial effects of DHPG is compatible with a developmental increase in eCB$_{Ca+GPCR}$. This suggests that increasing PLC activity might have a proportionately greater effect in neonates. We tested this prediction by applying a PLC activator (m-3M3FBS, 30 μM), with or without DHPG (1 μM) for 20 min in slices pretreated with agatoxin. For comparison, we also tested o-3M3FBS (30 μM), an inactive analog of m-3M3FBS, and observed that it had no effect on DSI in neurons recorded from either neonates (Fig. 6, A and I, n = 7) or juveniles (Fig. 6, E and I, n = 5). The active compound, m-3M3FBS applied for 20 min, had no effect on neonatal DSI at either 30 μM (Fig. 6, B and I, n = 5) or 100 μM (n = 3, data not shown). In contrast, in juveniles, DSI was increased from 26 ± 2% to 59 ± 5% by 30 μM m-3M3FBS (Fig. 6, E, F, and I, n = 5, P < 0.01 compared with the results from the o-3M3FBS-treated group). The higher concentration of m-3M3FBS (100 μM) had no further effect in juveniles (data not shown).

We then tested the ability of PLC enhancement to affect eCB$_{Ca+GPCR}$. DHPG continued to increase DSI in the presence of o-3M3FBS in neonates (16 ± 3% to 27 ± 3%; n = 17, P < 0.001, paired t-test, Fig. 6, A, C, and I) and juveniles (24 ± 4%
to 53 ± 7%, n = 6, P < 0.002, paired t-test; Fig. 6, E, G, and I; P < 0.01). When m-3M3FBS was coapplied with DHPG in neonates, DSI was enhanced from 23 ± 3% to 55 ± 7% (n = 6; P < 0.002); i.e., to the level of juvenile DSI in DHPG (Fig. 6, B, D, and I). This enhanced DSI effect was mediated via increasing PLC activity because it was blocked when U-73122 (5 μM) was coapplied with m-3M3FBS and DHPG for 20 min (17 ± 6% to 22 ± 9%, n = 7, P = 0.51, paired t-test; data not shown). On the other hand, m-3M3FBS did not affect the DHPG-induced DSI increase in juveniles, which went from 26 ± 4% to 54 ± 7% (n = 5, P < 0.01, paired t-test, Fig. 6, H and I). These results are consistent with the hypothesis that DHPG and m-3M3FBS act via a common pathway to increase eCB$_{Ca^{2+}GPCR}$. The differential effects of drugs acting on PLC support the conclusion that the developmental increase in eCB$_{Ca^{2+}GPCR}$ is related to upregulation of this combined pathway.

**DISCUSSION**

It has been proposed that the eCB system undergoes developmental upregulation from PN7 to PN21, and that this effect is attributable to a postsynaptic increase in eCB production (Zhu and Lovinger 2010). However, the mechanisms involved in this change have remained almost entirely unknown. Using a combination of whole cell voltage-clamp recording and Western blot analysis, we have investigated these issues in rats while expanding the age range being tested from PN1 to PN43. Our major findings are that, over the age range tested, 1) of the three known mechanisms of eCB mobilization, there were substantial changes in only the combined mechanism, eCB$_{Ca^{2+}GPCR}$, manifested as an enhancement of DSI caused by simultaneous stimulation of GPCRs, showed a large developmental increase. 2) The purely GPCR-dependent mechanism, eCB$_{GPCR}$, as assessed by application of mAChR or group I mGluR agonists alone, was largely ineffective in inducing eCB release. 3) Although we cannot rule out a minor effect, our data suggest that there is no significant change in functional CB1R levels, in agreement with the report of Zhu and Lovinger (2010).

While the full biochemical details of the eCB$_{Ca^{2+}GPCR}$ pathway have not been worked out, probably PLCβ (Hashimoto-tani et al. 2005) and diacylglycerol-α (Gao et al. 2010; Tanimura et al. 2010) are central components. We observed developmental increases in protein levels of both mGluR5 and PLCβ1, which are consistent with the proposed mechanism and, furthermore, that manipulations of PLCβ had age-dependent effects on eCB mobilization. The findings suggest that both of these factors contribute to the developmental differences in eCB$_{Ca^{2+}GPCR}$. This is the first report of developmental regulation of a molecular mechanism of eCB$_{Ca^{2+}GPCR}$ and could have important implications for understanding the role of
eCBs during this growth period. The fact that increases in the
synergistic mechanism occur suggests that eCBs could play a
role when pre- and postsynaptic activity are simultaneously
active in sculpting neuronal circuitry in the growing brain. This
possibility is discussed below.

**Molecular mechanism of developmentally enhanced eCB**

The eCB responsible for most signaling processes, including eCB
gPCR, is involved in the modulation of DSI by eCBCa, eCBGPCR,
and eCBCa-GPCR.

**Molecular mechanism of developmentally enhanced eCB:**

**Fig. 4.** Juveniles have significantly greater amounts of mGluR5 and phospholipase C (PLC) β1 protein than neonates. A: representative photomicrographs of Western blots of mGluR5 and PLCβ1 proteins from hippocampal slices after electrophysiological DSI recordings. Note two lanes labeled “18” for mGluR5 protein are from two different PN18 animals and illustrate the variability possible at this critical age. 

Importantly, there is no quantitative difference between maximally activated DHPG-enhanced DSI in neonates (with the PLC activator) and juveniles (with or without the PLC activator), which is consistent with a ceiling effect of PLC stimulation.

Our results suggest that m-3M3FBS potentiates the DHPG enhancement of DSI by increasing PLC activity, which is in agreement with studies showing that m-3M3FBS strongly enhances PLC-dependent superoxide-generating activity and stimulates production of inositol phosphates (Bae et al. 2003). Moreover, inhibition of PLC prevents m-3M3FBS-induced apoptosis in a human renal cell line (Jung et al. 2008). On the other hand, because m-3M3FBS can stimulate a transient increase in \( [Ca^{2+}]_i \) in neurons (Bae et al. 2003), we cannot totally exclude the possibility that part of the enhancement of eCBCa-GPCR by m-3M3FBS in neonates reflects an increase in \( [Ca^{2+}]_i \). Nevertheless, any increase in \( [Ca^{2+}]_i \), caused by m-3M3FBS would have had to be relatively minor, because DSI in m-3M3FBS was not enhanced in neonates, as it would have been if \( [Ca^{2+}]_i \) had been greatly elevated.

**Molecular mechanism of developmentally enhanced eCBCa-GPCR:**

**Fig. 5.** DHPG-mediated enhancement of DSI is prevented by coapplication of a PLC inhibitor, U-73122, in both neonates and juveniles. Representative time course is shown of eIPSCs recorded from CA1 neurons in the presence of DHPG plus either U-73122 (B and D), or its inactive analog, U-73343 (A and C), in slices obtained from neonatal and juvenile rats, as indicated. Traces below are averages of eIPSCs recorded before and during DSI and before and during bath application of the respective chemicals. E: histograms show that U-73122 prevented the DHPG-mediated potentiated response of DSI in both neonates and juveniles (unpaired t-tests, \( *P < 0.05 \)). Note DSI magnitude is greater in juveniles than in neonates in the presence of DHPG plus U-73343 (unpaired t-tests, \( *P < 0.05 \)). All the slices were preincubated with agatoxin before the DSI recordings.
is 2-arachidonoylglycerol (2-AG) rather than anandamide (see Kano et al. 2009 for review). PLC is directly involved in the synthesis of 2-AG, whereas anandamide, which mediates eCB signaling in certain circumstances (e.g., Ade and Lovinger 2007; Azad 2004; Chavez et al. 2010; Grueter et al. 2010; Kim and Alger 2010), does not require PLC. Our data are, therefore, consistent with the conclusion that developmental upregulation of 2-AG mobilization mediates the increase in eCBCa/GPCR.

CB1R and CCK interneurons are present in the hippocampus at birth (Morozov and Freund 2003a, 2003b), and DSI is present at an early neonatal stage (Bernard et al. 2005). Although CB1R mRNA expression levels gradually increase in the hippocampus between the fetal period and adulthood (Har-kany et al. 2007), our functional assays did not detect strong evidence for changes in CB1R levels. Compared with the eCBCa+GPCR, there were no substantial developmental changes in eCBCa or eCBGPCR, and, had functional CB1R levels been rising significantly, there would have been. Similarly, Zhu and Lovinger (2010) reported that, in hippocampal slices, eIPSCs response to bath application of the CB1R agonist, WIN55212-2, did not change over the similar developmental period between neonates and early juveniles, which is inconsistent with an increase in CB1Rs. This conclusion is further supported by our observation of a large increase in DSI when preceded by DHPG or CCh application; neither of these GPCRs affects CB1R levels. Finally, the PLC activator is unlikely to affect CB1R, but had significant effects on eCBs mobilization. Thus the increase in GPCR-enhanced DSI is most likely to be explained principally by increased eCB mobilization and not increased CB1R.

Fig. 6. A PLC activator potentiates the DHPG-enhanced DSI in neonates to the level of juveniles. Representative time course is shown of eIPSCs recorded from CA1 neurons in the presence of a PLC activator, m-3M3FBS (right, B, D, F, H) or its inactive analog, o-3M3FBS (left, A, C, E, G) in slices obtained from neonatal and juvenile rats, with or without DHPG coapplication as indicated. Traces below are representative averaged eIPSCs before and during bath application of the chemicals. I: histograms show that o-3M3FBS had no effect on basal DSI or the action of DHPG on DSI in either group (ANOVA, #P < 0.05; ##P < 0.01). m-3M3FBS significantly increased basal DSI in juveniles but not neonates (ANOVA, ##P < 0.01). When coapplied with DHPG, m-3M3FBS increased neonatal DSI to the juvenile level (ANOVA, #P < 0.05, ##P < 0.01); the juvenile level was not further enhanced by m-3M3FBS. All of the slices were preincubated with agatoxin. Calibration: continuous traces = 1 nA, 1 min; mean traces = 1 nA, 100 ms.
Lack of substantial changes in eCB$_{Ca}$ or eCB$_{GPCR}$. We found that, in the presence of agatoxin, DSI was greater in juveniles than in neonates, but that the difference between the two groups was much reduced compared with those in the absence of agatoxin. Agatoxin does not enhance eCB mobilization. The extracellular electrical stimulation excites both DSI-sensitive (CB1R-expressing) and DSI-insensitive (non-CB1R-expressing) interneurons. Thus agatoxin enhances the relative degree of DSI of eIPSCs by eliminating DSI-insensitive eIPSCs (Lenz et al. 1998).

The fact that agatoxin pretreatment reduced the magnitude of the developmental increase in DSI suggests that a shift in the balance of non-CB1R-expressing and CB1R-expressing interneurons probably takes place during this time period. If the percentage of CB1R-expressing neurons increases with development, the measured DSI would increase, even if the actual eCB$_{Ca}$ process was not much more effective in these animals. Detailed morphological analysis of the development of different types of interneurons in the hippocampus (Tricoire et al. 2011), showed that, from PN5 to PN30, interneurons lacking CB1R (including parvalbumin-expressing cells) always outnumber CB1R-expressing interneurons, but the ratio of non-CB1R-expressing to CB1R-expressing cells drops from PN5 to PN30. This implies that the ability of agatoxin to enhance DSI should be less in juveniles than neonates, which is what we found.

While we confirmed the developmental increase in DSI reported by Zhu and Lovinger (2010), our data suggest that this may not be entirely attributed to a true increase in eCB$_{Ca}$. The developmental decrease in non-DSI-sensitive eIPSCs could have affected their results as well, although they observed significant effects of 1-amino-1,3-dicarboxycyclopentane and CCh on eIPSCs in neonates, indicating the existence of other differences between the two studies. Their data do show an increase in eCB$_{Ca-GPCR}$ with 1-amino-1,3-dicarboxycyclopentane or CCh (Zhu and Lovinger 2010; Fig. 4), however, they do not comment on whether the relative effects of the GPCR agonists on DSI increased with age.

Functional significance of increases in eCB$_{Ca+GPCR}$. The synergistic interactions between DSI and mGluR or mAChR activation are probably not mediated by an increase in [Ca$^{2+}$], (Kim et al. 2002). Hashimoto etc et al. (2005) showed that eCB$_{GPCR}$ is absent in PLC${\beta}_{1}^{-/-}$ mice, whereas eCB$_{Ca}$ is unaffected by the knockout. This suggested that PLC$\beta$, which is both Ca$^{2+}$ and G protein dependent, serves as a “coincidence detector” for the two pathways of eCB mobilization. This simple hypothesis is somewhat controversial (e.g., Edwards et al. 2006), and Edwards et al. (2008) proposed instead that Ca$^{2+}$ actually primes the eCB$_{GPCR}$ pathway and the priming effect outlasts the actual period of increased [Ca$^{2+}$], i.e., that true temporal coincidence of the two signals is not required. This opens up the possibility that PLC$\beta$, although a key upstream regulator, might not actually be the molecular integrator. Regardless of the details, a synergistic interaction between Ca$^{2+}$ and the products of GPCR activation is an especially potent stimulus for eCB mobilization. We now report that development increases in PLC$\beta$ activity accompany the increased eCB$_{Ca+GPCR}$, yet, because protein levels of both mGluR5 and in PLC${\beta}_{1}$ occurred, upregulation of PLC${\beta}_{1}$ per se cannot be identified as the only mechanism.

However, the molecular details of this issue are ultimately resolved, our demonstration that development affects primarily the synergistic eCB$_{Ca+GPCR}$ pathway is itself quite significant. Unlike the purely postsynaptic Ca$^{2+}$-dependent eCB mechanism, or the purely GPCR-dependent eCB mechanism, the combined mechanism is equally dependent on both pre- and postsynaptic factors. This means that it is ideally suited to take part in the formation and shaping of critical neuronal circuitry, which requires coordinated presynaptic and postsynaptic activity (Shatz 1990). The near simultaneous occurrence of presynaptic release of glutamate and significant postsynaptic depolarization to activate voltage-gated Ca$^{2+}$ channels will result in a heightened mobilization of eCBs. If released near inhibitory CB1R-expressing synapses, the eCBs will suppress the release of GABA, thereby increasing net excitation in the neuronal circuits. Moreover, even weak excitatory synaptic activity, too weak to induce long-term potentiation on its own, if it occurs during DSI-induced IPSC depression, can undergo long-term potentiation (Carlson et al. 2002). Increased DSI caused by release of glutamate should be even more effective in this function. Thus the combination of strong postsynaptic and presynaptic activity could lead to the strengthening of synapses that are crucial for appropriate circuit development.

ACKNOWLEDGMENTS

We thank the members of the Alger and McCarthy laboratories for valuable discussion.

GRANTS

This work was supported by grants CMRPD1C0181 and CMRPD1C0182 (to S.-L. Liang) from Chang Gung Medical Research Foundation of Taiwan, ROC; and National Institutes of Health Grants R01-NS-050525-01 (to M. M. McCarthy) and R01-DA-014625 (to B. E. Alger).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


