Abnormal Synaptic Transmission in the Olfactory Bulb of Fyn-Kinase–Deficient Mice

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Abnormal synaptic transmission in the olfactory bulb of Fyn-kinase–deficient mice. J. Neurophysiol. 79: 137–142, 1998. We studied synaptic transmission in the granule cells in the olfactory bulb of the homozygous Fyn (a nonreceptor type tyrosine kinase)-deficient (fyn+/fyn−) and heterozygous Fyn-deficient (+/fyn−) mice by using slice preparations from the olfactory bulb. Stimulation to the lateral olfactory tract and/or centrifugal fibers to the olfactory bulb evoked field excitatory postsynaptic potentials (fEPSPs) in the granule cells. In +/fyn− mice, fEPSPs were augmented by bicuculline, a γ-aminobutyric acid (GABA A ) antagonist and picrotoxin, whereas fEPSPs in fyn+/fyn− mice were much less sensitive to bicuculline and picrotoxin. Application of D-2-amino-5-phosphonopentanoic acid had no effect on fEPSPs in both +/fyn− and fyn+/fyn− mice. (1S,3R)-1-aminoenecyclo-pentane-1,3-dicarboxylate, an agonist of metabotropic glutamate receptors caused a similar depression of fEPSPs in both +/fyn− and fyn+/fyn− mice. In +/fyn− mice tetanic stimulation to the lateral olfactory tract and/or centrifugal fibers induced N-methyl-d-aspartate (NMDA)-dependent long-term potentiation (LTP) of fEPSPs, whereas LTP was impaired in fyn+/fyn− mice. Our results demonstrate altered functions of GABA A receptors and impaired long-term potentiation in the olfactory bulb of Fyn-deficient mice.

INTRODUCTION

Fyn, a nonreceptor type tyrosine kinase of the Src family, is strongly expressed in the central nervous system (CNS), suggesting that it plays an important role in brain function. Fyn-deficient mutant mice have a variety of abnormal signs, such as impaired long-term potentiation in the hippocampus, with deficits in spatial learning (Grant et al. 1992), abnormality in suckling behavior in neonatal mutants (Yagi et al. 1993a), increased fearfulness and enhanced sensitivity to audiogenic seizures (Miyakawa et al. 1994, 1995).

An expression study in which β-galactosidase gene (lacZ) was inserted into fyn revealed that Fyn in neural tissues appears to be developmentally regulated (Yagi et al. 1993a,b, 1994). Staining by X-gal showed that high expression was observed in the olfactory tract, the olfactory epithelium, and optic tract in the late embryonic stage. In 7-day-old Fyn-deficient pups, the modified glomerular complex of the olfactory bulb was abnormal in shape and reduced in size. In adults a high level of fyn expression was observed in olfactory bulb, hippocampus, and cerebellum. These results implied an abnormal function of the olfactory system in Fyn-deficient mice. There has been, however, no report on olfactory synaptic transmission at the cellular level in Fyn-deficient mice.

The present experiment is aimed at studying possible changes in the olfactory system in Fyn-deficient mutant mice by using a slice preparation of the olfactory bulb. Because making slice preparations of the olfactory bulb connected to intact olfactory nerve is difficult, we have made slices of the olfactory bulb with the efferent and afferent fibers to the olfactory cortex. We have compared the synaptic potentials in the olfactory bulb of the homozygous Fyn-deficient (fyn+/fyn−) with those in heterozygous Fyn-deficient (+/fyn−) and wild type mice. We have also studied the effects of pharmacological agents acting on the glutamate and γ-aminobutyric acid (GABA) receptors, because many studies have reported glutamatergic and GABAergic transmission in the olfactory bulb (Hayashi et al. 1993; Jahr and Nicoll 1982; Trombley and Westbrook 1990, 1992; Van de Pol et al. 1995; Wells and Kauer 1994).

We have found reduced sensitivity to antagonists of GABA A receptors and impaired long-term potentiation in the olfactory bulb of Fyn-deficient mice.

METHODS

Fyn-deficient mice

Fyn-deficient mice were generated by inserting the β-galactosidase gene (lacZ) into the fyn gene as reported previously (Yagi et al. 1993b, 1994). In brief, TT2 cells, one of the mouse embryonic stem (ES) cell-lines from F1 embryos between C57BL/6 and CBA mouse strains, were mutated by electroporation of the targeting vector pHFZprNeoDT (a targeting vector for mouse Fyn locus), which is composed of BgII-Sphl DNA fragment of fyn with the exon 3 locus disrupted by insertion of β-galactosidase (lacZ) and neomycin resistant gene (Neo) and at the 3′ end added to diphtheria toxin fragment A gene (DT) for negative selection. G418 resistant homologous recombinant cells were selected and injected into eight cell stage embryos to deliver chimeric mice. Chimeric mice were crossed with C57BL/6, an inbred mouse strain, and heterozygous mice were generated. Heterozygous mice were crossed within themselves and homozygous mice were obtained by the Mendelian rule. By immunoblotting with anti-Fyn antibody, we have confirmed no Fyn protein in the olfactory bulb of the homozygotes. When we performed behavioral and anatomic analyses of Fyn mutant mice, we could not detect any abnormalities in the heterozygotes.
Preparation of the olfactory bulb slice

Mice were anesthetized by ether and decapitated. A block of brain containing the olfactory bulb and a part of the olfactory cortex was dissected out. The block was cut in half at the midline and placed on a plate in the slicer with cut surface down for making the slice. Sagittal sections of the olfactory bulb (400–500 μm width) were cut by a vibratome (Microslicer, Dosaka EM). Usually, slices containing medial part of the olfactory bulb (0.5–1 mm from the midline) were used. The slice was submerged in artificial cerebrospinal fluid containing (in mM) 127 NaCl, 1.5 KCl, 1.24 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHPO4, and 10 glucose, bubbled with 95% O2-5% CO2 and maintained at 36°C.

Electrophysiological recording

After 2 h of incubation a single slice was transferred to the recording chamber as described in previous work in the laboratory (Tsubokawa et al. 1994–1996). The chamber was continuously perfused with bathing solution maintained at 35.0 ± 0.5°C. Bipolar stimulating electrode of Teflon-coated silver wires (50-μm diameter tip) was placed on the near part of the lateral olfactory tract, which can easily be seen under the microscope. Glass pipette recording electrode containing five times Krebs without calcium and glucose was placed on a more rostral part of the olfactory bulb. Usually low frequency stimulation (200 μs, 100–150 μA) was applied at 0.08–0.12 Hz. Tetanic stimulation consisted of two trains of 100 pulses at 100 Hz separated by an interval of 20 s. To measure the maximal rate of rise of field excitatory postsynaptic potentials (fEPSP), records were digitized at 10 kHz and analyzed by an on-line computer. Groups of three successively evoked fEPSPs were averaged before calculating the maximal rate of change of potential within a time window selected around the rising phase.

RESULTS

Distribution of the field potential in the olfactory bulb

In total, 85 mice (29 fyn+/fyn−, 37 +/+ fyn−, and 19 wild type) were used as subjects. No difference was found in the data from wild type and those from +/+ fyn− mice. Schematic diagram of a sagittal section of the olfactory bulb slice is shown in Fig. 1A. We chose the slices containing medial part of the olfactory bulb because relatively stable responses were obtained in these preparations. Stimulating electrode placed near the center of the slice could activate both the lateral olfactory tract (LOT) and centrifugal fibers (CF), inducing various forms of field potentials in the olfactory bulb. It is generally observed that negative field potentials were evoked near the dorsal and the middle part of the olfactory bulb, whereas positive potentials prevailed in the ventral part. Typical examples of field potentials in +/+ fyn− are shown in Fig. 1B. Paired stimuli with the interval of 40 ms were applied at a fixed point and the recording electrode was moved along dorsoventral axis. The late negative field potentials with a marked facilitation were recorded in the dorsal part of the slice (Fig. 1Ba). Amplitude of the negative

FIG. 1. Distribution of field excitatory postsynaptic potentials (fEPSP) recorded from olfactory bulb slice. A: schematic diagram of sagittal section of olfactory bulb slice. Stimulating electrode is placed near center of olfactory bulb where lateral olfactory tract (LOT) and centrifugal fibers (CF) run. a–f: position of recording electrode. b and c: distances of 500 and 1000 μm from stimulating electrode, respectively. Distance between each recording position is 500 μm. B: representative fEPSP traces in a Fyn (a nonreceptor type tyrosine kinase)-deficient hetero (+/+ fyn−) mouse. Paired pulse stimulations with interval of 40 ms were applied. C: similar sample fEPSP traces as in B but from a Fyn-deficient homo (fyn−/fyn−) mouse. D: example record of field potentials in another +/+ fyn− mouse. Potentials are composed of early negative potentials (∗) followed by late negative potentials. Note that paired stimuli with an interval of 40 ms produce a marked facilitation in late potentials but early negative potentials are unchanged.
measured the amplitude of fEPSPs evoked by the same stimulus intensity (200 μs, 115 ± 5 μA) with a fixed distance between stimulating and recording electrode (500 μm), the peak negative amplitude was 1.21 ± 0.18 mV (12 slices, n = 12) in +/-fyn* mice, whereas it was 0.66 ± 0.12 mV (16 slices, n = 16) in fyn*/fyn* mice, showing a significant difference [P < 0.05, analysis of variance (ANOVA)].

In our slice preparations, stimulation could activate both LOT and CF to the olfactory bulb neurons and evoked field potentials generally consisted slow negative potentials but in some cases they were preceded by brief negative potentials. Figure 1D shows such an example came from another +/-fyn* mouse. The early negative potentials (*) could be either antidromic action potentials in the mitral cells or passing fiber potentials of LOT or CF. When paired pulse stimulation with intervals of 30–100 ms were applied the late negative potentials consistently showed facilitation, while the early negative potentials were unchanged. This supports the view that the late negative potentials are excitatory postsynaptic potentials (EPSPs) originated from the granule cells.

**Effects of antagonists of GABA<sub>A</sub> and glutamate receptors**

It was reported that granule cells in the olfactory bulb receive GABAergic and glutamatergic synaptic inputs (Jahr and Nicoll 1982; Mori and Kishi 1982; Trombley and Westbrook 1990; Wellis and Kauer 1994). We investigated effects of antagonists of GABA<sub>A</sub> receptors and glutamate receptors on fEPSPs in the olfactory bulb slices. Figure 2 shows the sequence of maximum slopes of fEPSPs under the action of a series of antagonists. The maximal rate of rise of fEPSP was determined as described in METHODS.

The maximal slope was measured within a time window selected around the rising phase of fEPSPs so as to exclude the presynaptic volleys (insets). In the slice of +/-fyn* mouse (Fig. 2A), application of bicuculline (25 μM) with picrotoxin (30 μM) greatly potentiated fEPSPs. After washing out the GABA<sub>A</sub> antagonist application of APV, an antagonist of N-methyl-D-aspartate (NMDA) receptor produced no appreciable change in fEPSPs, whereas CNQX, a non-
NM DA antagonist almost completely blocked fEPSPs. The results indicate that fEPSPs are mainly generated by activation of non-NMDA receptors in the granule cells. In contrast to +/+ fyn mice, fyn+/fyn mice were much less sensitive to GABA A antagonist, as shown in Fig. 2B. Application of bicuculline and picrotoxin in the same concentration as in +/+ fyn mice gave no potentiation of fEPSPs in fyn+/fyn mice. APV was ineffective and CNQX suppressed fEPSPs in the same way as in +/+ fyn mice. The results suggest that GABAergic rather than glutamatergic transmission is disturbed in fyn+/fyn mice. The average maximum slopes of fEPSPs after applying bicuculline and picrotoxin increased to 123.4 ± 3.1% in +/+ fyn mice (n = 12) but to 104 ± 4.7% in fyn+/fyn mice (n = 9).

We tested paired stimulation to the slices treated with bicuculline and picrotoxin. Paired pulse facilitation was consistently seen in the presence of GABA A antagonist both in +/+ fyn and fyn+/fyn mice, indicating that the mechanism of paired pulse facilitation was not impaired in Fyn-deficient mice.

**Effects of agonists of metabotropic glutamate receptors**

We next examined the effects of agonists of metabotropic glutamate receptors on fEPSPs in the olfactory bulb, because the expression of metabotropic glutamate receptors (mGluRs) have been documented (Hayashi et al. 1993; Tromley and Westbrook 1990; Van Del Pol 1995). Application of (1S,3R)-ACPD (100 μM) in +/+ fyn mice effectively suppressed fEPSPs (Fig. 3A). The peak amplitude of fEPSPs decreased to about 60% of the control but soon recovered after washing out the drug (5 slices, n = 5). Similar reversible suppression by (1S,3R)-ACPD was seen in fyn+/fyn mice (7 slices, n = 7; Fig. 3B). By contrast, application of L-2-amino-4-phosphonobutyric (L-AP4; 100 μM) did not produce any appreciable change either in +/+ fyn (5 slices, n = 5) or fyn+/fyn mice (2 slices, n = 2; data not shown). Because (1S,3R)-ACPD selectively activates mGluR 2,3 and L-AP4 activates mGluR 4,6,7, suppression of fEPSPs by (1S,3R)-ACPD could be caused by activation of mGluR 2 or mGluR 3 but not by mGluR 4,6,7. The results also indicate that mGluR 2 or mGluR 3 receptors in the olfactory bulb are not modified in Fyn-deficient mice.

**Long-term potentiation in the olfactory bulb**

When tetanic stimulation (2 trains of 100 pulses of 100 Hz) was applied to CF fibers, fEPSPs in +/+ fyn mice showed potentiation of fEPSPs. The magnitude of potentiation was 110–130% of the control and it lasted for more than 30 min, exhibiting a long-term potentiation (LTP; Fig. 4A). When we applied APV (100 μM) to the bathing solution, control low-frequency stimulation (0.08 Hz) gave no appreciable change in fEPSPs and the tetanic stimulation failed to elicit LTP (Fig. 4B). In contrast to the slices from +/+ fyn mice slices of fyn+/fyn mice failed to show LTP of fEPSPs (Fig. 4C). Tetanic stimulation induced only transient potentiation and fEPSPs returned to the original level in 10 min. Profile of changes in the slope for fEPSP after tetanus in fyn+/fyn mice was similar to that in +/+ fyn treated with APV.

**DISCUSSION**

**Origin of field potentials in the olfactory bulb slice**

In the present study, stimulation on the slice preparation could excite both afferent and efferent fibers of the olfactory bulb (Fig. 5). The afferents (CF), which consist of fibers
from the piriform cortex, anterior olfactory nucleus, anterior commissure, and nucleus diagonal band make numerous synapses with the granule cells at various sites (Mori 1987; Shepherd 1972). The efferent fibers are axons of mitral/tufted (M/T) cells forming LOT. The antidromic activation of mitral cells may excite granule cells through the dendrodendritic and recurrent axon collateral synapses. We concluded that field potentials in the olfactory bulb originated from EPSPs in the granule cells for following reasons. First, paired pulse stimulation consistently produced facilitation of the negative field potentials, which are characteristic of the postsynaptic potentials with little contribution of antidromic responses. Second, it was reported that activation of the mitral cells by paired stimuli caused depression (paired pulse depression) of the granule cells through the reciprocal dendrodendritic synapses between the mitral cell and the granule cell (Jahr and Nicoll 1982; Mori 1987; Mori and Takagi 1978). Third, (1S,3R)-ACPD, an agonist of mGluR2 inhibited fEPSPs both in +/fyn+ and fyn-/fyn- mice. By using the accessory olfactory bulb (AOB) of rat, Hayashi et al. (1993) reported that mGluR2 activation could reduce GABA release from the granule cell to the mitral cell, which in turn could augment synaptic activation of the granule cells. Although AOB is different from the olfactory bulb in the present study, it is probable that inhibitory effect of (1S,3R)-ACPD could be caused by similar mechanism and dendrodendritic synapses contribute little, if any, in generation of fEPSPs. The field potentials in the olfactory bulb slice in the present study differed from in vivo experiments where LOT/CF stimulation generated both antidromic activation of M/T cells and orthodromic responses in granule cells (Mori 1987; Nakashima et al. 1978; Rall and Shepherd 1968; Shepherd 1972). Possibly, most of M/T cells are damaged in the process of taking out the olfactory bulb by cutting out the connecting olfactory nerves.

Because fEPSPs were not affected by APV but were nearly completely blocked by CNQX, they are mostly composed of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor-mediated response. Inhibition of granule cell responses by the glutamate receptor antagonists was reported in rat (Jacobson and Hamberger 1986) and in salamander (Wellis and Kauer 1994) olfactory bulbs.

In fyn+/fyn- mice, the amplitude of fEPSPs was generally smaller than that in +/fyn+ mice. In a previous report on gross anatomy of fyn+/fyn- mice, no apparent abnormality was observed in the olfactory bulb by hematoxilin-eosin staining, although detailed histological study has not been done (Yagi et al. 1993b). However, in 7-day-old pups of Fyn-deficient mutant, a reduced size of the modified glomerular complex (MGC) was observed (Yagi et al. 1993a). One possible explanation for the smaller amplitude of fEPSPs in fyn+/fyn- mice is that deficiency of Fyn, which is essential for normal formation of the receptor molecules caused functional impairment during development in fyn+/fyn- mice.

Reduced sensitivity to GABA_A receptor antagonists in Fyn-deficient mice

In +/fyn+ mice, fEPSPs were potentiated after applying bicuculline and picrotoxin indicating that GABA_A receptors in the granule cells were blocked. In contrast, fEPSPs in fyn+/fyn- mice were much less sensitive to antagonists of GABA_A receptors. The granule cells are known to receive inhibitory inputs from neighboring granule cells and other interneurons (Mori 1987; Shepherd 1972; Wellis and Kauer 1994). Therefore the reduced sensitivity of fEPSPs in fyn+/fyn- mice to the antagonists could be caused by a reduction in the number of GABA_A receptors in the granule cells. Alternatively, the mutual inhibitory transmission between the granule cells or GABAergic transmission from the short axon cells may be dysfunctional. Similar paired pulse facilitation of fEPSPs was seen in both +/fyn+ and fyn+/fyn- mice and this facilitation was not modified by GABA_A antagonists. This suggests that the mechanism of facilitation was not impaired in Fyn-deficient mice.

Long-term potentiation in the olfactory bulb slice

So far, LTP in the olfactory system was reported by Strippling and colleagues (Patneau and Strippling 1992; Strippling et al. 1991) in in vivo study by using chronically implanted electrodes. They suggested that LTP may represent an enhancement of inhibitory interactions with the piriform cortex and between cortex and the olfactory bulb. In the slice of rat piriform cortex, Kanter and Haberly (1990) found that LTP in the piriform cortex was blocked by APV, indicating that it is NMDA dependent. In the present study, LTP of fEPSPs in the granule cells of the olfactory bulb was found to be NMDA receptor-dependent. Because APV did not affect fEPSPs with low-frequency stimulation, but blocked LTP, induction of LTP in the granule cells is triggered by activation of the NMDA receptor as in many other systems (Bliss and Collingridge 1993). In relation to the function of NMDA receptors of Fyn-deficient mice, Miyakawa et al. (1997) found that fyn+/fyn- mice were more sensitive to ethanol when measuring the duration of the loss of the righting reflex and they observed loss of acute tolerance...
to ethanol inhibition of NMDA-mediated EPSPs in CA1 pyramidal neurons in \textit{fyn}^{-/-}fyn^{-/-} mice.

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