Cell-Specific Differential Expression of Na⁺-Channel β 1-Subunit mRNA in the Olfactory System during Postnatal Development and after Denervation

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Activity-dependent mechanisms have been implicated in olfactory system development but, although such activity requires ion channels, few reports have described their expression in the olfactory system. We investigated the developmental and denervation-induced regulation of the Na⁺-channel β 1 subunit (Na β 1) in rat olfactory bulb (OB) and piriform cortex (PC). *In situ* hybridization shows that Na β 1 mRNA expression is upregulated developmentally, but with different time courses in mitral, tufted, and pyramidal cells. In mitral cells, label was detected at postnatal day 4 (P4) and gradually increased to P14. Tufted cells were devoid of Na β 1 mRNA before P14, when most cells expressed adult levels. In pyramidal cells of PC, Na β 1 expression was not detectable clearly until P14, with maximal expression at P28.

To examine the regulation of Na β 1 mRNA, we surgically deafferented the OB at P30 and compared the effects on Na β 1

with those for Na⁺-channel α -subunit (Na α) mRNAs. Within 5 d of surgery, the Na β 1 and Na α II signals within tufted cells disappeared almost completely. Na β 1 and Na α II expression was decreased in mitral cells to low-to-moderate levels. In pyramidal cells, Na β 1 mRNA expression was decreased moderately without significant changes in Na α II mRNA. Deafferentation had no detectable effects on Na α I or III mRNAs in either OB or PC. These data indicate that Na β 1 mRNA is expressed differentially in subpopulations of cells in the olfactory system during development and after deafferentation and suggest that the expression of Na β 1 is regulated independently of Na α mRNAs via cell-specific and pathway-specific mechanisms.

Key words: *in situ* hybridization; mRNA; Na⁺-channel subunits; deafferentation; olfactory bulb; piriform cortex; mitral cells; tufted cells; pyramidal cells

Voltage-dependent Na⁺ channels, which consist of heterotrimeric complexes of α , β 1, and β 2 subunits (Na α , Na β 1, Na β 2) (Catterall, 1986), are important determinants of neuronal function. Although Na α subunits alone are sufficient to encode functional channels (Noda et al., 1986b), Na β 1 appears to alter functional expression, voltage dependence, and inactivation kinetics of Na⁺ channels (Isom et al., 1994). Spatial and temporal expression patterns of Na α and Na β 1 mRNAs are observed in the developing rat CNS. Na α I, Na α II, and Na α III_A account for >85% of Na⁺ channels in most regions of adult rat brain, whereas Na α III is expressed primarily in fetal and neonatal animals (Brysch et al., 1991; Black et al., 1994). Na β 1 mRNA is upregulated during postnatal development in rat brain (Patton et al., 1994; Sashihara et al., 1994a). The late expression of Na β 1 and Na β 2 in rat retinal ganglion cells (Wollner et al., 1988) has raised the possibility that their appearance is a rate-limiting step in the development of mature Na⁺ channels. Thus, expression of Na⁺-channel β subunits may play a significant role in CNS development by conferring critical physiological characteristics needed for normal function of cells (Isom et al., 1994).

Recent studies have shown that various mechanisms link neuronal excitation to alterations in gene expression. For example, several studies have described the expression of transcription factors after alteration of sensory input in the spinal cord (Hunt et al., 1987; Wisden et al., 1990), suprachiasmatic nucleus (Aronin et al., 1990), somatosensory cortex (Mack and Mack, 1992), and olfactory bulb (OB) (Guthrie et al., 1993). Similarly, deafferentation can regulate the expression of specific genes and associated proteins transsynaptically (Stone et al., 1991).

In OB, innervation by olfactory receptor neurons (ORNs) or odor-induced afferent activity can influence transcriptional regulation and functional protein synthesis of several neuroactive compounds. For example, deafferentation of OB after chemical or surgical destruction of ORNs reduces the level of dopamine, tyrosine hydroxylase (TH), and TH mRNA (Nadi et al., 1981; Baker et al., 1983; Ehrlich et al., 1990). Substance P (Kream et al., 1984) and cholecystokinin (Scarlsbrick and Gall, 1988) expression can be reduced similarly in juxtglomerular neurons. In addition, a complementary upregulation of glomerular layer dopamine (spiperone, D2) (Brunjes et al., 1985) and nerve growth factor-binding sites can be shown in deafferented OB (Gomez-Pinilla et al., 1989). Related studies have shown reorganization of higher olfactory areas such as piriform cortex (PC) during development (for review, see Brunjes and Frazier, 1986) and after removal of OB projections (Westrum, 1980; Friedman and Price, 1986a; Westrum and Bakay, 1986; Westenbroek et al., 1988).

Deafferentation reduces neuronal activity throughout OB (Benson et al., 1984) and increases the susceptibility of mitral or tufted

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cells to local-circuit inhibition (Wilson and Wood, 1992). The presence of Na⁺-dependent action potentials in the dendrites and axons of OB and PC primary neurons (Mori, 1987; Haberly, 1990; Trombley and Shepherd, 1992) raises the possibility that Na⁺-channel subunit composition contributes to both the development of olfactory system organization and its response to deafferentation. To address the question of whether the afferent innervation of OB by ORNs can regulate Na⁺-channel gene expression transsynaptically, we have used *in situ* hybridization to study Na β 1 expression during normal postnatal development and after surgical deafferentation of OB. Using subtype-specific probes, we compared changes in Na β 1 and Na α mRNA expression induced by deafferentation to identify cell type-specific alterations in the expression of the genes for both subunits.

MATERIALS AND METHODS

Animals. Timed-pregnant Sprague–Dawley rats were maintained with access to food and water *ad libitum*. Rats ($n = 6$ at each age) were designated as postnatal day 0 (P0) on the day of parturition and studied at P4, P7, P14, P28, and P47.

Probes. Na α - and Na β 1-specific probes were generated as described previously (Black et al., 1994; Oh et al., 1994a,b; Waxman et al., 1994). Briefly, for Na α I, -II, and -III mRNA, plasmids pBSNC1, -2, and -3 were constructed carrying 3'-noncoding regions of rat brain Na α I, -II (Noda et al., 1986a), and -III (Kayano et al., 1988) cDNA; these regions correspond to nucleotide sequences 7385–7820 (I), 6807–7302 (II), and 6325–6822 (III) (numbering sequence according to GenBank), respectively. The inserts were sequenced by the dideoxy chain-termination method and were specific for Na α I, -II, and -III. Plasmid pNa β 1, containing a 334 bp fragment corresponding to nucleotides 457–790 (coding region of Na β 1), was constructed as described previously (Oh et al., 1994b).

From these cloned plasmids for Na α and Na β 1, digoxigenin-labeled single-strand RNA probes were prepared using a DIG RNA-labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. To generate antisense probes, HindIII-cleaved pBSNC1, EcoRI-cleaved pBSNC2, BamHI-cleaved pBSNC3, and HindIII-cleaved pNa β 1 were used as templates for transcription with T3 or T7 RNA polymerase. To generate sense probes, XbaI-cleaved pBSNC1, BamHI-cleaved pBSNC2, EcoRI-cleaved pBSNC3, and SacII-cleaved pNa β 1 were used as templates for transcription with T3 or T7 RNA polymerase. The reaction products were purified using Chroma Spin-100 column (Clontech, Cambridge, UK).

Tissue preparation and *in situ* hybridization. The protocol of Oh et al. (1994b) was used for *in situ* hybridization with minor modifications. The rats were anesthetized deeply by CO₂ narcosis and perfused transcardially with PBS (4°C) followed by 4% *p*-formaldehyde in 0.14 M Sorensen's phosphate buffer (pH 7.4, 4°C). Tissues were removed, immersed in the fixative for 2–4 hr at 4°C, transferred to 4% *p*-formaldehyde and 30% sucrose in 0.14 M phosphate buffer, and stored overnight at 4°C. Twelve micrometer cryostat sections were mounted on poly-L-lysine-coated slides, desiccated overnight under vacuum, and sequentially incubated in the following: (1) fixative solution for 5 min; (2) PBS, three changes, 2 min each; (3) proteinase K (2.5 μ g/ml) in 100 mM Tris/50 mM EDTA, pH 8.0, for 25 min at 37°C; (4) PBS, three changes, 2 min each; (5) 0.1 M triethanolamine (TEA), pH 8.0, for 2 min; (6) 0.25% acetic anhydride in 0.1 M TEA for 10 min; (7) 2 $\times$ SSC, three changes, 2 min each, (20 $\times$ SSC stock solution: 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0); (8) prehybridization solution containing 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, and 100 μ g/ml salmon sperm DNA (Sigma) for 2 hr at 58°C; and (9) hybridization solution containing 50% formamide, 10% dextran sulfate, 5 $\times$ SSC, 1 $\times$ Denhardt's, 100 μ g/ml salmon sperm, and 0.5 ng/ μ l probe overnight at 58°C. The cRNA probes were incubated for 10 min at 65°C before adding them to hybridization solution. On each section, 30 μ l of freshly prepared hybridization solution was applied, covered with Parafilm, and incubated in a humidified chamber. After hybridization, slides were incubated sequentially in the following: (1) 4 $\times$ SSC for 5 min; (2) 2 $\times$ SSC, two changes, 10 min each; (3) RNase A (25–50 μ g/ml) in 10 mM Tris/500 mM NaCl, pH 7.5, for 1 hr at 37°C; (4) 2 $\times$ SSC, two changes, 10 min each; (5) 0.2 $\times$ SSC, three changes, 20 min each at 58°C; (6) buffer 1 (100 mM Tris/150 mM NaCl, pH 7.5) for 2 min; (7) blocking solution containing 2% sheep serum and 1% bovine serum

albumin in buffer 1 for 30 min; (8) anti-digoxigenin antibody conjugated to alkaline phosphatase (diluted 1:500–1:1000 in blocking solution, Boehringer) for 2 hr, (9) buffer 1, three changes, 5 min each; (10) buffer 2 (100 mM Tris/150 mM NaCl/50 mM MgCl₂, pH 9.5), three changes, 5 min each; and (11) color substrate solution containing 420 μ g/ml nitro blue tetrazolium and 188 μ g/ml 5-bromo-4-chloro-3-indolyl-phosphate in buffer 2 overnight. The color reaction was stopped by several changes of 10 mM Tris/1 mM EDTA, pH 8.0. Sections were dehydrated, cleared, and coverslipped with Permount.

In addition to examination of sections hybridized with sense-strand probes, control experiments established the specificity of the probes and *in situ* hybridization protocol: (1) hybridization of sections without addition of a probe, (2) pretreatment of sections with RNase A, and (3) use of adult rat liver, which does not express Na⁺ channels, as a control (Isom et al., 1992; Oh et al., 1994a,b). These control experiments did not reveal any specific labeling.

In deafferentation studies, paired coronal sections through OB and PC (one each from a control and treated animal) were placed on the same slide to control for potential differences in hybridization conditions.

OB denervation. Sprague–Dawley rats, 30 d postnatal ($n = 12$), were anesthetized with pentobarbital (Nembutal) at 65 mg/kg, i.p. A small craniotomy was performed unilaterally immediately caudal to the cribriform plate, over the rostral pole of the OB. Using a small pipette (tip diameter of $\sim$ 250 μ m) under direct visual control with a microscope, suction was used to transect the ORN axons. When the procedure was completed, the resulting cavity was packed with gelfoam and the skin was sutured. After survival times of either 5, 7, or 14 d, the subjects were killed and assessed with *in situ* hybridization as described above. Representative tissue sections were stained with cresyl violet for histological correlation. Successful denervation of the OB was confirmed at the time of killing and dissection.

RESULTS

Antisense RNA probes complementary to the 3'-noncoding region of Na α (I, II, III) and coding regions of Na β 1 were used to determine the cellular sites of mRNA expression in OB and PC of rat brain by *in situ* hybridization histochemistry. Probe sequences were identical to those described previously by Waxman et al. (1994) (Na α I, -II, and -III) and Oh et al. (1994b) (Na β 1). No specific labeling was observed in sections hybridized with sense-strand control probes.

Localization of Na⁺-channel β 1-subunit mRNA in the developing olfactory system

The development of the rat olfactory system extends significantly into the postnatal period. For the developmental study of Na β 1 gene expression in OB and PC, we analyzed sections of rats from P4 through young adult. Specific labeling of Na β 1 mRNA in mitral, tufted, and pyramidal cells clearly increased with age (Figs. 1, 2). Our findings demonstrate that Na β 1 mRNA was upregulated in a cell-specific manner during later periods of olfactory system development.

Specific labeling of Na β 1 mRNA within OB was observed exclusively in mitral and tufted cells (Fig. 1). Na β 1 mRNA was just detectable at P4 in mitral cells (Fig. 1A, arrowhead), and levels of Na β 1 gradually increased in these cells through P14 (Fig. 1B,C, arrowheads). The level of Na β 1 mRNA in the P28 through young adult mitral cells was slightly lower than that in the P14 cells (Fig. 1D, arrowhead).

In tufted cells, expression of Na β 1 mRNA was not apparent at P4 and P7 (Fig. 1A,B). Specific labeling was observed by P14 and persisted into adulthood in most cells (Fig. 1C,D, arrows). These results indicate that there is a difference in the time course of Na β 1 mRNA expression between mitral and tufted cells. Interestingly, no detectable labeling was observed in developing interneurons, such as periglomerular and granule cells that modify the activity of the projection neurons via local-circuit interactions (Fig. 1).

Figure 1. Increased expression of Na β 1 mRNA during postnatal development in OB. Sections of OB at P4 (A), P7 (B), P14 (C), and P28 (D) are shown. At P4 in mitral cells, labeling is present and is increased up to P14 (arrowheads), whereas in tufted cells labeling is not detectable until P14 but increases through P28 (arrows). E, External plexiform layer; G, glomerular layer; M, mitral cell layer. Scale bars, 100 μ m.

Figure 2. Increased expression of Na β 1 mRNA during postnatal development in PC. Coronal sections of PC at P7 (*A*), P14 (*B*), P28 (*C*), and P47 (*D*) are shown. At P7 in pyramidal cells in layers II and III, no detectable labeling is observed. Beginning at P14, labeling in pyramidal cells in these layers is observed and increased up to P28 (arrowheads indicate layer II pyramidal cells; arrows indicate layer III pyramidal cells). No detectable changes in the expression of Na β 1 mRNA are observed at P47 compared with P28. 1, Layer I of PC; 2, layer II; 3, layer III. Scale bar, 50 μ m.

The expression of Na β 1 mRNA in both layer II and layer III pyramidal cells of PC was delayed compared with that in principal cells of OB. No detectable hybridization signal was observed in these cells at P7 (Fig. 2A). Weak but distinct expression signal was discerned at P14 (Fig. 2B, *arrowhead*) and had increased to reach adult levels by P28 (Fig. 2C,D, *arrowheads* and *arrows*). Layer I of PC was devoid of Na β 1 mRNA-expressing cells (Fig. 2).

Effect of deafferentation on Na β 1 and Na α mRNAs in OB and PC

In view of the age-dependent and cell-specific regulation of Na β 1 gene expression in developing OB and PC, we examined whether the expression of Na β 1 mRNA could be altered by reduction in neuronal activity *in vivo* (Figs. 3, 4). For comparison, and to determine whether alterations in Na β 1 mRNA expression were specific or were part of a generalized alteration in expression of all Na⁺-channel subunit mRNAs, we also examined the effect of such deafferentation on Na α I, -II, and -III mRNAs (Figs. 5, 6). A total of 12 animals was studied, 3 each for each of the four probes (Na α I, Na α II, Na α III, and Na β 1); the results were consistent across animals. The surgical denervation of OB causes a loss of primary afferent sensory inputs to OB and has been used to study the role of input–target interactions in the olfactory system. Decreased sensory input to OB is believed generally to cause a coordinated diminution in OB input to the pyramidal neurons of PC. The most striking observation in this study was that expression of Na α II and Na β 1 mRNA completely disappeared in tufted cells (Figs. 3, 5); none of the tufted cells expressed Na β 1 mRNA within 5 d after surgery (Fig. 3B,D). In contrast, in mitral cells deafferentation produced a slight decrease in the number of cells expressing Na α II and Na β 1 mRNA (13.8 ± 0.31 cells/200 μ m vs 11.2 ± 0.30 cells/200 μ m, respectively) as well as a decrease in the intensity of labeling (Figs. 3D, 5D, *arrowheads*); both of these mRNAs were found to be expressed at low-to-moderate levels in mitral cells after denervation. Thus, we have demonstrated that there is a difference in response to deafferentation (as well as a developmental difference) between mitral and tufted cells, although Na α II and Na β 1 mRNA appear to be regulated coordinately in each of these cell types after deafferentation.

In PC, expression of Na β 1 mRNA was decreased moderately in pyramidal cells within 5 d after surgical deafferentation (Fig. 4B,D, *arrowheads* and *arrows*), although Na α II mRNA expression was not affected significantly (Fig. 6D). Na α I and III, which were expressed at much lower levels than Na β 1 or Na α I (Figs. 5, 6), were unchanged in principal cells of both OB and PC (data not shown). Thus, it is unlikely that alterations in the expression of Na β 1 or Na α I reflect the loss of subpopulations of neurons in either OB or PC. Finally, in PC it appears that the expression of Na α II and Na β 1 genes is regulated differentially and/or is dependent on afferent input to pyramidal cells.

DISCUSSION

Our *in situ* hybridization observations demonstrate cell-specific developmental sequences of Na β 1 mRNA expression in rat olfactory system (Figs. 1, 2) and show that the expression of Na⁺-channel subunits in mitral and tufted cells (Figs. 3, 5) and in PC pyramidal neurons (Figs. 4, 6) is altered by OB deafferentation. The present results provide the first demonstration of transsynaptic modulation of Na β 1 mRNA expression after deafferentation.

Unilateral odor deprivation causes markedly reduced size and functional activity of the ipsilateral OB (Benson et al., 1984;

Brunjes, 1985, 1994). After deafferentation, the expression of Na β 1 mRNAs disappeared in tufted cells in the external plexiform layer and decreased slightly in mitral cells. This observation adds to a growing body of evidence segregating mitral and tufted cells (Greer, 1987; Greer and Halasz, 1987). Tufted cells are excited more readily by afferent input than mitral cells (Schneider and Scott, 1983). This may reflect differential synaptic input or more fundamental differences in membrane properties of mitral and tufted cells. Either mechanism could contribute to the observed differential downregulation of Na⁺-channel subunits. Alternatively, loss of primary afferent axons from OB may have trophic effects on mitral and tufted cells that cause alterations in Na⁺ mRNA, independent of alterations in excitation. Although such nonspecific mechanisms could contribute to our results in OB, it appears to be a less likely alternative for the pyramidal cells of PC, in which axonal inputs remained intact.

Although the current study focuses on the differential response of OB projection neurons, previous studies have demonstrated deafferentation-induced downregulation of other mRNA/proteins in juxtglomerular neurons (Nadi et al., 1981; Baker et al., 1983; Kream et al., 1984; Stone et al., 1991). Thus, neurons of OB generally may be sensitive to deafferentation, differing only in the expression of their response. Although deafferentation generally may cause a downregulation of protein synthesis, this is not a global effect. Indeed, as noted above, several receptors upregulate in OB after deafferentation, and glutamic acid decarboxylase is stable in several subpopulation neurons after deafferentation of OB (Stone et al., 1991). Our studies do not detect Na⁺-channel subunits in OB interneurons. This is consistent with previous reports of tissue- and cell-specific expression of Na⁺-channel subunits elsewhere in the CNS (Black et al., 1994). Granule cells and periglomerular cells are capable of generating action potentials (Mori, 1987). One possibility is that our probes are specific to Na⁺-channel subtypes expressed in larger projection neurons but not in smaller interneurons. Alternatively, the level of detection of our *in situ* hybridizations may not have been adequate to detect lower levels of mRNA that may be present in the populations of interneurons. Although there may be a correlation between neuron size and the degree of the DIG histochemical reaction, it is unlikely because low levels of signal were observed readily in mitral, tufted, and pyramidal neurons during development. In the same context, it could be argued that the complete loss of signal from tufted cells after denervation is a reflection of cell size and sensitivity of the histochemical detection method. This cannot be ruled out completely in the absence of quantitative data that could be obtained with radioactive probes. This open question, however, does not detract from our observations on the developmental time frame for Na⁺-channel subunit expression in projection neurons; nor does it temper our novel findings of afferent innervation regulation of Na⁺-channel subunit expression in OB and transsynaptic regulation in PC.

A vast majority of mitral cells in control tissue expressed Na β 1 mRNA, although the intensity of the histochemical reaction product was highly variable. Only a small proportion of tufted cells was observed to express Na β 1 mRNA. These cells appeared to correspond primarily to the middle tufted cell population, and there was little or no evidence of labeling among deep or superficial tufted cells. Although the proportion of middle tufted cells expressing label is unknown, it is clear that there was considerable variability in the intensity of the reaction product, as was observed among the labeled mitral cells. Between the populations of labeled mitral and tufted cells, there was overlap in the intensity of cell staining.

Figure 3. Denervation-induced downregulation of Naβ1 mRNA expression in OB. *A, B*, Lower-magnification micrographs show that Naβ1 mRNA is expressed at adult levels in P35 rat control mitral and tufted cells (*A*), whereas Naβ1 mRNA disappears in tufted cells and is decreased in ipsilateral mitral cells after 5 d of denervation (*B*). *C, D*, Higher-magnification micrographs showing control (*C*) and denervated (*D*) OBs. *Arrow* and *arrowheads* in *C* and *D* indicate Naβ1-expressing tufted cells and mitral cells, respectively. *E, F*, Cresyl violet-stained sections of control (*E*) and denervated (*F*) OBs. *O*, Olfactory nerve layer; *G*, glomerular cell layer; *E*, external plexiform layer; *M*, mitral cell layer; *GR*, granule cell layer. Scale bars: *A, B*, 300 μ m; *C, D*, 100 μ m; *E, F*, 200 μ m.

Figure 4. Denervation-induced downregulation of Na β 1 mRNA expression in PC. *A, B*, Lower-magnification micrographs show that Na β 1 mRNA is expressed at adult levels in P35 rat control pyramidal cells in layers II and III (*A*), whereas Na β 1 mRNA is decreased dramatically in layers II and III 5 d after denervation (*B*). *C, D*, Higher-magnification micrographs of PC showing control (*C*) and 5 d after denervation (*D*). Arrowheads and arrows in *C* and *D* indicate Na β 1-expressing pyramidal cells in layers II and III, respectively. Scale bars: *A, B*, 200 μ m; *C, D*, 100 μ m.

The effect of downregulation of Na⁺-channel subunits on the function of tufted, mitral, and pyramidal neurons is not known. Changes in the level and distribution of Na⁺ channels can cause

significant alterations in cellular activity (Mandel, 1992; Stys et al., 1993; Turner et al., 1993). For example, denervation of skeletal muscle causes a decrease in Na⁺-channel subunit expression, an

Figure 5. Denervation-induced downregulation of Na α II mRNA expression in OB. Sections of OB hybridized with Na α I (*A*), Na α II (*C*, *D*), and Na α III (*B*) antisense cRNA probes are shown. *A*, Labeling is observed in control mitral cells (*arrowhead*). *B*, Labeling is observed in both control mitral and tufted cells (*arrow* and *arrowhead*, respectively). Denervation had no apparent effects on expression of Na α I and Na α III mRNAs in these cells (data not shown). *C*, Na α II mRNA is expressed significantly in control mitral and tufted cells (*arrow* and *arrowhead*, respectively). *D*, Alternatively, Na α II mRNA disappears in tufted cells and is decreased in mitral cells (*arrowhead*) after 5 d of denervation. Scale bar, 100 μ m. Designations as in Figure 1.

accompanying increase in membrane resistance, and the appearance of fibrillation potentials (Buckingham, 1989; Yang et al., 1991). Similarly, suppression of channel function is sufficient to block action potentials and decrease conduction velocity (Stys et

al., 1992; Yokota et al., 1994). Conversely, upregulation of Na⁺-channel subunits in hyperexcitable phenotypes such as epilepsy appears to underlie, in part, the pathophysiology of these diseases (Sashihara et al., 1992). Thus, the present observations appear

Figure 6. Lack of effect of denervation on Na α I–III mRNA expression in PC. Coronal sections of PC hybridized with Na α I (*A*), Na α II (*B*, *D*), and Na α III (*C*) antisense cRNA probes. *A*, Weak Na α I labeling is observed in layer II and III control pyramidal cells (*arrow* and *arrowhead*, respectively). *B*, Significant Na α II labeling is observed in layer II and III control pyramidal cells (*arrow* and *arrowhead*, respectively). *C*, Weak Na α III labeling is observed in layer II and III control pyramidal cells (*arrow* and *arrowhead*, respectively). Denervation had no apparent effects on Na α I (data not shown), Na α II, or Na α III (data not shown) mRNA expression. *D*, *Arrow* and *arrowhead* indicate Na α II mRNA-expressing pyramidal cells in layers II and III. Scale bar, 50 μ m. Designations as in Figure 2.

generally to be consistent with the diminution in overall metabolic activity in OB that accompanies naris closure (Benson et al., 1984). However, the extent to which naris closure influences the activity of single neurons has not been studied extensively. Wilson and Wood (1992) reported that rat pups with naris occlusions from P1 to P20 showed little evidence of altered bulb activity until 40 d, when mitral cell inhibition increased. Downregulation of Na⁺-channel activity, because of the loss of subunits in the projection neurons as observed in the present study, could underlie the increase in susceptibility to inhibition.

Developmental upregulation of Na β 1 mRNA in the olfactory system

Na β 1 protein and mRNA in rat brain are upregulated progressively during the first 3 weeks after birth (Sutkowski and Catterall, 1990; Patton et al., 1994). Similarly, the present study shows that Na β 1 transcripts accumulate during postnatal development in the olfactory system (Figs. 1, 2). The increasing levels of Na β 1 mRNA during this time could arise from activation of the Na β 1 gene, from increased stability of the mRNA, or from increased processing of primary transcription products to mRNA. Scheinman et al. (1989) demonstrated that Na α expression is regulated at the level of gene transcription in developing rat forebrain. Further elucidation of the entire sequence linked to the regulation of expression may provide evidence regarding mechanisms involved in transneuronal regulation of ion channels.

The projection of ORNs to OB reflects a spatial and functional topography (Greer et al., 1981; Mori et al., 1985; Ressler et al., 1994; Vassar et al., 1994). Normal laminar segregation of afferent fibers in PC depends on axodendritic interactions during postnatal development (Friedman and Price, 1986b), which is consistent with the idea that function in the maturing olfactory system is regulated by sensory input via action potentials and synaptic transmission after birth. We observed different time courses of Na β 1 mRNA expression in mitral, tufted, and pyramidal cells. In principal cells of OB, our data suggest that Na β 1 mRNA expression reached adult levels by P14, with tufted cells showing slightly delayed expression compared with mitral cells (see Fig. 1). The slightly delayed Na β 1 mRNA maturation in tufted cells is consistent with their time of origin [embryonic day 16 (E16) to E19] compared with mitral cells (E14–E17) (Bayer, 1986). Thus, it could be argued that Na β 1 mRNA expression is related to the chronological age of the cell. However, in pyramidal cells of PC that originate at ~E14–E18, Na β 1 mRNA expression was delayed further until P28 (Fig. 2), consistent with the period of physiological maturation in PC (Schwob et al., 1984). These data suggest that Na β 1 mRNA expression is associated more closely with development of olfactory function induced by ORN input than with chronological age of the cells.

Deafferentation-induced downregulation of Na β 1 and Na α II mRNAs in OB and of Na β 1 mRNA in PC

Previous studies have demonstrated alterations in the expression of Na α mRNAs, including marked upregulation of Na α III, caused by axotomy in dorsal root ganglion neurons (Waxman et al., 1994) and facial motor neurons (Iwahashi et al., 1994). The present data demonstrate that deafferentation-induced sensory deprivation is followed by marked cell-specific changes in Na β 1 and Na α II mRNA expression in the olfactory system (see Figs.

3–6). Mitral, tufted, and pyramidal cells generate rapidly rising, tetrodotoxin-sensitive Na⁺ spikes (Mori, 1987). However, before the present study, little was known about subunit composition of voltage-sensitive Na⁺ channels in OB and PC, or about whether mechanisms exist in the olfactory system for regulation of Na⁺-channel expression.

The expression of Na β 1 mRNA was downregulated in all cell types that produce the transcripts after 5 d of deafferentation (see Figs. 3, 4), suggesting that maintenance of Na β 1 mRNA levels is dependent, at least in part, on a transsynaptic influence dependent on ORN input. The downregulation of the Na β 1 mRNA in PC pyramidal neurons occurred independent of any substantive structural alterations because of the loss of axons, as is occurring in OB with our denervation procedures. By comparison, Na α II mRNA exhibited downregulation in principal cells of OB (Fig. 5) but no change in pyramidal cells of PC (see Fig. 6D), suggesting that monosynaptic and disynaptic inputs have different effects on Na α II gene expression or that other PC inputs are capable of sustaining Na α II expression in pyramidal neurons. Alternatively, it should be noted that although the mitral and tufted cells of OB were deprived of all excitatory input, PC was not affected equivalently. Residual mitral and tufted cell spontaneous activity, although likely to be diminished significantly, would continue to provide some excitatory input to PC pyramidal neurons, as would the cortico-cortical association axons of layer Ib of PC. Consequently, an alternative interpretation would be that the Na β 1 mRNA level is more sensitive to deafferentation than Na α II mRNA.

There is evidence for regulatory feedback between spontaneous electrical activity and Na α expression *in vivo* and *in vitro* (Offord and Catterall, 1989; Dargent and Courand, 1990; Sashihara et al., 1994b). This modulation may allow neurons to maintain an appropriate density of Na⁺ channels and to adjust this density in response to external stimuli. The present results suggest that the pathway for this feedback is different from that involved in transsynaptic regulation of Na⁺-channel subunit expression. Lack of transsynaptic activity caused a downregulation of Na α II mRNA in OB but no change in PC, suggesting that the regulation of Na α I, Na α II, Na α III, and Na β 1 mRNA expression is a complex process that is both cell- and pathway-specific. Because Na β 1 has multiple effects on Na⁺-channel function (increased peak current, accelerated activation and inactivation, and altered voltage dependence of inactivation) (McClatchey et al., 1993; Isom et al., 1994), deafferentation may lead to altered excitability of neurons attributable to downregulation of Na α II and/or Na β 1 (Scholfield, 1980). In the olfactory system, there may be convergent mechanisms for transsynaptic regulation of neuronal excitability together with regulatory effects attributable to spontaneous electrical activity or constitutive metabolic activity in various cell types.

REFERENCES

- Aronin N, Sagar SM, Sharp FR, Schwartz WJ (1990) Light regulates expression of a FOS-related protein in rat suprachiasmatic nuclei. *Proc Natl Acad Sci USA* 87:5959–5962.
- Baker H, Kayano T, Margolis FL, Joh TH (1983) Transneuronal regulation of tyrosine hydroxylase expression in olfactory bulb of mouse and rat. *J Neurosci* 3:69–78.
- Bayer SA (1986) Neurogenesis in the rat primary olfactory cortex. *Int J Dev Neurosci* 4:251–271.
- Benson TE, Ryugo DK, Hinds JW (1984) Effect of sensory deprivation on the developing mouse olfactory system: a light and electron microscopic morphometric analysis. *J Neurosci* 4:638–653.

- Black JA, Yokoyama S, Higashida H, Ransom BR, Waxman SG (1994) Sodium channel mRNA I, II and III in the CNS: cell-specific expression. *Mol Brain Res* 22:275-289.
- Brunjes PC (1985) Unilateral odor deprivation: time course of changes in laminar volume. *Brain Res Bull* 14:233-237.
- Brunjes PC (1994) Unilateral naris closure and olfactory system development. *Brain Res Rev* 19:146-160.
- Brunjes PC, Frazier LL (1986) Maturation and plasticity in the olfactory system of vertebrates. *Brain Res Rev* 11:1-45.
- Brunjes PC, Smith-Crafts LK, McCarty R (1985) Unilateral odor deprivation: effect on the development of olfactory bulb catecholamines and behavior. *Dev Brain Res* 22:1-6.
- Brysch W, Creutzfeldt OD, Luno K, Schlingensiepen R, Schlingensiepen KH (1991) Regional and temporal expression of sodium channel messenger RNAs in the rat brain during development. *Exp Brain Res* 86:562-567.
- Buckingham ME (1989) The control of muscle gene expression: a review of molecular studies on the production and processing of primary transcripts. *Br Med Bull* 45:608-629.
- Catterall WA (1986) Molecular properties of voltage-sensitive sodium channels. *Annu Rev Biochem* 55:953-985.
- Dargent B, Courand F (1990) Down-regulation of voltage-dependent sodium channels initiated by sodium influx in developing neurons. *Proc Natl Acad Sci USA* 87:5907-5911.
- Ehrlich ME, Grillo M, Joh TH, Margolis FL, Baker H (1990) Transneuronal regulation of neuron specific gene expression in the mouse olfactory bulb. *Mol Brain Res* 7:115-122.
- Friedman B, Price JL (1986a) Age-dependent cell death in the olfactory cortex: lack of transneuronal degeneration in neonates. *J Comp Neurol* 246:20-31.
- Friedman B, Price JL (1986b) Plasticity in the olfactory cortex: age-dependent effects of differentiation. *J Comp Neurol* 246:1-19.
- Gomez-Pinilla F, Guthrie KM, Leon M, Nietro-Sampedro M (1989) NGF receptor increase in the olfactory bulb of the rat after early odor deprivation. *Dev Brain Res* 48:161-165.
- Greer CA (1987) Golgi analyses of dendritic organization among denervated olfactory bulb granule cells. *J Comp Neurol* 257:442-452.
- Greer CA, Halasz N (1987) Plasticity of dendrodendritic microcircuits following mitral cell loss in the olfactory bulb of the murine mutant Purkinje cell degeneration. *J Comp Neurol* 256:284-298.
- Greer CA, Stewart WB, Kauer JS, Shepherd GM (1981) Topographical and laminar localization of 2-deoxyglucose uptake in rat olfactory bulb induced by electrical stimulation of olfactory nerves. *Brain Res* 217:279-293.
- Guthrie KM, Anderson AJ, Leon M, Gall C (1993) Odor-induced increases in *c-fos* mRNA expression reveal an anatomical "unit" for odor processing in olfactory bulb. *Proc Natl Acad Sci USA* 90:3329-3333.
- Haberly LB (1990) Olfactory cortex. In: *The synaptic organization of the brain*, 3rd Ed (Shepherd GM, ed), pp 317-345. New York: Oxford UP.
- Hunt SP, Pini A, Evan G (1987) Induction of c-Fos-like protein in spinal cord neurons following sensory stimulation. *Nature* 328:632-634.
- Isom LL, De Jongh KS, Catterall WA (1994) Auxiliary subunits of voltage-gated ion channels. *Neuron* 12:1183-1194.
- Isom LL, De Jongh KS, Patton BF, Reber FX, Offord J, Charbonneau H, Walsh K, Goldin AL, Catterall WA (1992) Primary structure and functional expression of the β 1 subunit of the rat brain sodium channel. *Science* 256:839-842.
- Iwahashi Y, Furuyama T, Inagaki S, Morita Y, Takagi H (1994) Distinct regulation of sodium channel types I, II and III following nerve transection. *Mol Brain Res* 22:341-345.
- Kayano T, Noda M, Flockerzi V, Takahashi H, Numa S (1988) Primary structure of rat brain sodium channel III deduced from the cDNA sequence. *FEBS Lett* 228:187-194.
- Kream RM, Davis BJ, Kayano T, Margolis FL, Macrides F (1984) Substance P and catecholaminergic expression in neurons of the hamster main olfactory bulb. *J Comp Neurol* 222:140-154.
- Mack KJ, Mack PA (1992) Induction of transcription factors in somatosensory cortex after tactile stimulation. *Brain Res* 12:141-147.
- Mandel G (1992) Tissue specific expression of the voltage-sensitive sodium channel. *J Membr Biol* 125:193-205.
- McClatchey AI, Cannon SC, Slaughter SA, Gusella JF (1993) Cloning and expression of a sodium channel β 1 subunit from human brain. *Hum Mol Genet* 2:745-749.
- Mori K (1987) Membrane and synaptic properties of identified neurons in the olfactory bulb. *Prog Neurobiol* 29:275-320.
- Mori K, Fujita SC, Imamura K, Obata K (1985) Immunohistochemical study of subclasses of olfactory nerve fibers and their projection to the olfactory bulb in rabbit. *J Comp Neurol* 242:214-229.
- Nadi NS, Head R, Grillo M, Hempstead J, Grannot-Reisfeld N, Margolis FL (1981) Chemical deafferentation of the olfactory bulb: plasticity of the level of tyrosine hydroxylase, dopamine and norepinephrine. *Brain Res* 213:365-371.
- Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima H, Kurahashi M, Takahashi II, Numa S (1986a) Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* 320:188-192.
- Noda M, Ikeda T, Suzuki T, Takeshima H, Takahashi T, Kuno M, Numa S (1986b) Expression of functional sodium channels from cloned cDNA. *Nature* 322:826-828.
- Offord J, Catterall WJ (1989) Electrical activity, cAMP, and cytosolic calcium regulate mRNA encoding sodium channel α subunits in rat muscle cells. *Neuron* 2:1447-1452.
- Oh Y, Black JA, Waxman SG (1994a) The expression of rat brain voltage-sensitive Na⁺ channel mRNAs in astrocytes. *Mol Brain Res* 23:57-65.
- Oh Y, Sashihara S, Waxman SG (1994b) *In situ* hybridization localization of the Na⁺-channel β 1-subunit mRNA in rat CNS neurons. *Neurosci Lett* 176:119-122.
- Patton DE, Isom LL, Catterall WA, Goldin AL (1994) The adult rat brain β 1 subunit modifies activation and inactivation gating of multiple sodium channel α subunits. *J Biol Chem* 269:17649-17655.
- Ressler KJ, Sullivan SL, Buck LB (1994) Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell* 79:1245-1255.
- Sashihara S, Oh Y, Black JA, Waxman SG (1994a) Expression of Na⁺ channel β 1-subunit mRNA in rat central nervous system. *Soc Neurosci Abstr* 20:64.
- Sashihara S, Yanagihara N, Izumi F, Murai Y, Mita T (1994b) Differential upregulation of voltage-dependent Na⁺ channels induced by phenytoin in brains of genetically seizure-susceptible (E1) and control (ddY) mice. *Neuroscience* 62:803-811.
- Sashihara S, Yanagihara N, Kobayashi II, Izumi F, Tsuji S, Murai Y, Mita T (1992) Overproduction of voltage-dependent Na⁺ channels in the developing brain of genetically seizure-susceptible E1 mice. *Neuroscience* 48:285-291.
- Scarlsbrick IA, Gall CM (1988) Regulation of CCK and NPY immunoreactivity within the adult rat olfactory bulb by primary afferent input. *Soc Neurosci Abstr* 14:477.
- Scheinman RI, Auld VJ, Goldin AL, Davidson N, Dunn RJ, Catterall WA (1989) Developmental regulation of sodium channel expression in the rat forebrain. *J Biol Chem* 254:10660-10666.
- Schneider SP, Scott JW (1983) Orthodromic response properties of rat olfactory bulb mitral and tufted cells correlate with their projection pattern. *J Neurophysiol* 50:358-378.
- Schwob JE, Haberly LB, Price JL (1984) The development of physiological responses of the piriform cortex in rats to stimulation of the lateral olfactory tracts. *J Comp Neurol* 223:223-237.
- Scolfield CN (1980) Time-course of declining electrical activity in guinea-pig olfactory cortex after olfactory removal. *Neurosci Lett* 19:297-301.
- Stone DM, Grillo M, Margolis FL, Joh TH, Baker H (1991) Differential effect of functional olfactory bulb deafferentation on tyrosine hydroxylase and glutamic acid decarboxylase messenger RNA levels in rodent juxtglomerular neurons. *J Comp Neurol* 311:223-233.
- Stys PK, Ransom BR, Waxman S (1992) Tertiary and quaternary local anesthetics protect CNS white matter from anoxic injury at concentrations that do not block excitability. *J Neurophysiol* 67:236-240.
- Stys PK, Sontheimer H, Ransom B, Waxman S (1993) Noninactivating, tetrodotoxin-sensitive Na⁺ conductance in rat optic nerve axons. *Proc Natl Acad Sci USA* 90:6976-6980.
- Sutkowski EM, Catterall WA (1990) β 1 subunits of sodium channels: studies with subunit-specific antibodies. *J Biol Chem* 265:12393-12399.
- Trombley PQ, Shepherd GM (1992) Noradrenergic inhibition of synaptic transmission between mitral and granule cells in mammalian olfactory bulb cultures. *J Neurosci* 12:3985-3991.
- Turner RW, Meyers DER, Barker JL (1993) Fast pre-potential generation in rat hippocampal CA1 pyramidal neurons. *Neuroscience* 53:949-959.
- Vassar R, Chao SK, Sitcheran R, Nunez JM, Vossall LB, Axel R (1994) Topographic organization of sensory projections to the olfactory bulb. *Cell* 79:981-991.

- Waxman SG, Kocsis JD, Black JA (1994) Type III sodium-channel mRNA is expressed in embryonic but not adult spinal sensory neurons, and is reexpressed following axotomy. *J Neurophysiol* 72:466–470.
- Westenbroek RE, Westrum LE, Hendrickson AE, Wu JY (1988) Ultrastructure of synaptic remodeling in piriform cortex of adult rats after neonatal olfactory bulb removal. *J Comp Neurol* 274:334–346.
- Westrum LE (1980) Alterations in axons and synapses of olfactory cortex following olfactory bulb lesions in newborn rats. *Anat Embryol* 160:153–172.
- Westrum LE, Bakay RAE (1986) Plasticity in the rat olfactory cortex. *J Comp Neurol* 243:195–206.
- Wilson DA, Wood JC (1992) Functional consequences of unilateral olfactory deprivation: time course and age sensitivity. *Neuroscience* 49:183–192.
- Wisden W, Errington ML, Williams S, Dunnett SB, Waters C, Hitchcock D, Evan G, Bliss TVP, Hunt SP (1990) Differential expression of immediate-early genes in the hippocampus and spinal cord. *Neuron* 4:603–614.
- Wollner DA, Sheinman RI, Catterall WA (1988) Sodium channel expression and assembly during development of retinal ganglion cells. *Neuron* 1:727–737.
- Yang S, Sladky JT, Kallen RG, Barchi RL (1991) TTX-Sensitive and TTX-insensitive sodium channel transcripts are independently regulated in adult skeletal muscle after denervation. *Neuron* 1:727–737.
- Yokota T, Saito Y, Miyatake T (1994) Conduction slowing without conduction block of compound muscle and nerve action potentials due to sodium channel block. *J Neurol Sci* 124:220–224.