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Neuregulin1 and ErbB expression in the uninjured and regenerating olfactory mucosa

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Abstract

Neuregulin1, a protein involved in signaling through the ErbB receptors, is required for the proper development of multiple organ systems. A complete understanding of the expression profile of Neu-regulin1 is complicated by the presence of multiple isoform variants that result from extensive alternative splicing. Remarkably, these numerous protein products display a wide range of divergent functional roles, making the characterization of tissue-specific isoforms critical to understanding signaling. Recent evidence suggests an important role for Neuregulin1 signaling during olfactory epithelium development and regeneration. In order to understand the physiological consequences of this signaling, we sought to identify the isoform-specific and cell type-specific expression pattern of Neu-regulin1 in the adult olfactory mucosa using a combination of RT-qPCR, FACS, and immunohistochemistry. To complement this information, we also analyzed the cell-type specific expression patterns of the ErbB receptors using immunohistochemistry. We found that multiple Neuregulin1 isoforms, containing predominantly the Type I and Type III N-termini, are expressed in the uninjured olfactory mucosa. Specifically, we found that Type III Neuregulin1 is highly expressed in mature olfactory sensory neurons and Type I Neuregulin1 is highly expressed in duct gland cells. Surprisingly, the divergent localization of these Neuregulin isoforms and their corresponding ErbB receptors does not support a role for active signaling during normal turnover and maintenance of the olfactory mucosa. Conversely, we found that injury to the olfactory epithelium specifically upregulates the Neuregulin1 Type I isoform bringing the expression pattern adjacent to cells expressing both ErbB2 and ErbB3 which is compatible with active signaling, supporting a functional role for Neuregulin1 specifically during regeneration.

Keywords

Neuregulin1; ErbB; Olfactory epithelium; Isoform analysis

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1. Introduction

Neuregulin1 (Nrg1) signaling through the ErbB receptor family is fundamental to the development of multiple organ systems in vertebrates (Meyer and Birchmeier, 1995; Meyer et al., 1997; Britsch et al., 1998; Wolpowitz et al., 2000; Li et al., 2002). In order to convey specificity to Nrg1 signaling for its diverse developmental roles, the gene is subject to rich alternative splicing resulting in numerous (15–20) tissue- and function-specific protein products (Fig. 1a). Although all of the protein products function as ligands for ErbB receptors (specifically ErbB3 and ErbB4), the process by which this occurs is dramatically different between splice variants and heavily impacts downstream signaling.

Overall, alternative exon selection occurs in 3 broad regions of the *Nrg1* gene: the 5' region, the EGF domain, and the 3' region—with each domain containing multiple exon and splice variant options (Buonanno and Fischbach, 2001) (Fig. 1a). For the N-terminal isoforms, alternative splicing in the 5' region of *Nrg1* selects the first exon of the protein, Type I, II, or III, and is responsible for dictating whether the resulting protein is secreted or membrane-bound. The protein products of Type I and Type II *Nrg1* are single-pass transmembrane proteins containing cell surface trafficking information on either their C-terminal intracellular domain (Wen et al., 1992; Liu et al., 1998a,b), or their N-terminus (Peles and Yarden, 1993; Burgess et al., 1995), in addition to an extracellular proteolysis domain that allows for extracellular release (Montero et al., 2000). These proteins are capable of paracrine signaling with ErbB receptors expressed on nearby cell surfaces and are released as diffusible signals. Conversely, the protein product of Type III *Nrg1* is a double-pass transmembrane protein that undergoes extracellular proteolytic processing to expose a membrane-tethered signaling domain that is only capable of juxtacrine signaling with receptors located on neighboring cells (Wang et al., 2001). This juxtacrine signaling has been studied at length in the nervous system, where Type III Nrg1 expressed on neuronal axons interacts with ErbB3 expressed on neighboring Schwann cells to promote Schwann cell maturation and maintenance (Shah et al., 1994; Dong et al., 1995; Garratt et al., 2000). In addition to canonical receptor signaling, Type III Nrg1 isoforms containing an “a” tail domain are also capable of acting as a receptor (Bao et al., 2003), although few biological examples of this bidirectional signaling have been reported (Bao et al., 2004).

Along with these distinct functional differences that arise from N-terminal isoforms of Nrg1, there are 2 other variable regions that are susceptible to rich alternative splicing and can influence downstream function: the EGF domain and the C-terminal intracellular domain (ICD). The EGF domain is necessary and sufficient to bind and activate ErbB receptors, and a complete EGF domain consists of a canonical region (cEGF) spliced to one of three carboxy motifs: α , β , or γ (Holmes et al., 1992). Differences in the carboxy-motif relate to binding and activation efficiency, with β isoforms displaying the highest proclivity for both (Wen et al., 1994; Pinkas-Kramarski et al., 1996). The addition of a short “stalk” region immediately following the EGF domain allows for increased proteolytic processing and release of soluble Type I or membrane-tethered Type III ligands (Montero et al., 2000). Finally, differences in the C-terminal ICD have an apparent difference only in their capacity for bidirectional signaling, as discussed above with the Type III isoform, although it remains to be determined whether future studies will uncover further functionality.

Here, we aim to identify the Nrg1 isoforms that are specific to the adult olfactory mucosa and the cell-type localization of these isoforms as well as their cognate ErbB receptors. Olfactory tissue is unique in its neuroregenerative capacity, wherein it is able to replenish both neuronal and non-neuronal cell types in response to injury. Given the known roles of Nrg1 and ErbB signaling throughout development, and particularly within the nervous system, we are interested in using this tissue to identify specific Nrg1 isoforms and signaling mechanisms that may function in an environment of continued neuronal regeneration. The architecture of the mucosa supports the possibility for both secreted and/or membrane-bound forms of Nrg1. The mucosa includes an underlying lamina propria with mesenchymal elements that can dictate the growth of the olfactory epithelium through secretion of signaling molecules, similar to what is seen with Nrg1 Type I guidance of heart formation (Kramer et al., 1996; Wadugu and Kuhn, 2012). Additionally, because the epithelium is richly populated with neurons that must project to and synapse at the olfactory bulb, the lamina propria contains a population of olfactory ensheathing cells (OECs) that bundle the projecting axons together, much like Schwann cells in the nervous system.

In addition to a permissive architecture, there is physical evidence for both types of proteins as well. Our lab has generated data suggesting that Nrg1 is found in conditioned media (CM) from a lamina propria-derived cell line, supporting the possibility of a secreted form of Nrg1 in the lamina propria (unpublished data). Additionally, Nrg1 has been reported in the rat olfactory epithelium, although isoform-specificity and cellular localization have not been fully elucidated (Salehi-Ashtiani and Farbman, 1996). Nrg1 has also been shown to be expressed in mouse olfactory bulb and ensheathing cells; however, these expression patterns do not change significantly after injury (Williams et al., 2004). Finally, early experiments have verified the presence of EGFR and ErbB2 as well as the absence of ErbB4 in the olfactory epithelium, suggesting active Nrg1 signaling, but this work is largely incomplete, providing little information on cell type-specification and expression patterns following injury (Holbrook et al., 1995; Salehi-Ashtiani and Farbman, 1996; Vullhorst et al., 2009).

Here, we not only confirm the presence of both secreted and membrane-bound Nrg1 in the mucosa, but also characterize for the first time the entire repertoire of Nrg1 isoforms expressed throughout this tissue. Additionally, by focusing on specific cell types, we are able to show how the spatial expression pattern for Nrg1 and the ErbB family members changes following injury. Ultimately, this provides a detailed survey of the complete Nrg1 signaling network in olfactory tissue and suggests that Nrg1/ErbB signaling has an important role in olfactory epithelium regeneration.

2. Results

2.1. Characterization of Nrg1 isoform expression in the uninjured vs injured olfactory mucosa

We first aimed to characterize the entire repertoire of exons expressed in the olfactory mucosa using qRT-PCR primers specifically designed to amplify each possible exon variant. The uninjured epithelial and lamina propria layers were physically separated to look individually at each tissue compartment (Fig. 1B, C). The results indicate that multiple isoforms are expressed in complex patterns across tissue and cell type within the mucosa

(Fig. 1B,C-black bars). Having established this baseline for *Nrg1* exon expression in the uninjured murine olfactory mucosa, we next sought to understand whether the predominant patterns of expression seen in these tissues are altered after injury. Here, we used methyl bromide (MeBr), an olfactotoxic gas, to induce olfactory mucosal injury, which activates the progenitor cell populations and initiates regeneration (Schwob et al., 1995). After enzymatic separation of the epithelial and lamina propria layers, we extracted RNA from exposed animals 2 days post MeBr lesion (dpl) and analyzed exon variance using qRT-PCR. These results show that exon expression and preference are significantly altered after injury, suggesting that modulation of *Nrg1* isoforms may play a role in the regenerative response, especially in the olfactory epithelium (Fig. 1B, C).

A detailed analysis of these isoform-specific changes reveals several diverging but consistent trends. With respect to the 5' region, which determines the mechanism by which *Nrg1* associates with its receptor, the Type III exon is the predominant isoform found in both the epithelium and lamina propria and changes little following injury. The next highest abundant isoform in epithelium is Type I which exhibits a significant increase after injury. Similarly, the next most abundant in lamina propria is Type I, but its expression is significantly downregulated after injury. With respect to the EGF variable domain, which reportedly modulates ligand/receptor binding efficiency, there is a complex pattern of expression that requires further investigation. When the data are analyzed as fold changes post-injury, this domain shows a striking inverse relationship between epithelium and lamina propria exon regulation. Finally, with respect to the 3' terminal region, which regulates intracellular trafficking and bidirectional signaling, post-injury epithelium shifts from a preference for the "a" over the "b" terminus to nearly equal expression of both. The lamina propria also expresses a preference for the "a" terminus but this preference is unchanged after injury.

Together, these data provide a detailed catalog of exons that are expressed in the olfactory mucosa. To characterize the identity and abundance of full-length isoforms, lamina propria and epithelium cDNA samples from uninjured adult mice were PCR-amplified using forward primers specific to either the Type I or Type III start exon paired with reverse primers specific to either the C-terminal "a" or "b" ICD, resulting in 4 possible primer pairs (Supplementary Fig. 1A). Based on the results from Fig. 1, the minimal expression of the Type II, γ -EGF, and "c" isoforms warranted exclusion from this deeper analysis. Amplified PCR products were cloned into the TOPO® vector and ten positive colonies were sequenced from each reaction to determine the identity of the corresponding internal EGF domain (Supplementary Fig. 1B–D). The Type 1b primer set did not generate a clonable product, suggesting that this splice variant does not exist in the entire olfactory mucosa. The Type Ia variant is restricted to the lamina propria, while the Type IIIa and b variants exist in both the lamina propria and epithelium. However, regardless of flanking variations, the predominant internal sequences are overwhelmingly β 1-EGF. This corroborates the previous qRT-PCR observations that lamina propria and epithelium both contained predominantly β 1-EGF. In more detail, Type Ia, which is only found in the lamina propria, contains 60% β 1-, 20% α -, and interestingly, 20% of neither β - nor α -EGF, but rather the cEGF domain spliced directly to the canonical transmembrane (TMC) domain—a splice variant that has not been reported

in the literature (Supplementary Fig. 1B, gray). Type IIIa variants are conserved between lamina propria and epithelium, with the β 1-EGF fragment almost exclusively linked with Type III. This is also seen in Type IIIb variants where the epithelium expresses 100% β 1-EGF compared to 70% in the lamina propria. The remaining 30% in the lamina propria consists of 10% β -EGF and 20% α -EGF (Supplementary Fig. 1C,D). Our exon analysis in Fig. 1 reveals the presence of β 3-EGF in the uninjured epithelium, however that could not be analyzed here because the β 3-EGF variants are truncated immediately following the stalk domain and lack a terminal ICD region for primer binding.

Finally, although results from Fig. 1C (green) show that there are low levels of Type I (7%) in the epithelium, we were unable to amplify enough product with the “a” or “b” reverse primers to acquire positive clones after TOPO[®] cloning. This is likely attributable to the much higher sensitivity of qRT-PCR compared to the clonal analysis described above. To address this issue, we grew unbiased cultures of the entire TOPO[®] reaction and PCR-amplified the DNA using a forward primer specific to cEGF and reverse primers specific for the “a” or “b” ICD. Using this technique, we confirmed that Type I exists only with the “a” ICD (Supplementary Fig. 1E). Conversely, in order to identify the splicing on the N-terminus of Type I variants, we PCR-amplified cDNA with a Type I forward primer and α -EGF or β -EGF reverse primers. These results indicate that Type I exists only with the α -EGF domain (Supplementary Fig. 1F). In summary, these data confirm the presence of 9–10 distinct Nrg1 isoforms in the olfactory mucosa with specific localization within either epithelial or lamina propria compartments (Fig. 1D, E).

2.2. Nrg1 shows cell-type selective expression patterns in the uninjured olfactory mucosa

While we have described the global distribution of Nrg1 iso-forms in the epithelial and lamina propria compartments, we sought to more accurately understand the cell type-specific expression patterns using FACS-purified subpopulations of the olfactory mucosa. Using a variety of fluorescent reporter mouse strains and antibodies, we sorted and isolated RNA from 8 populations of cells: lamina propria fibroblasts, olfactory ensheathing cells (OECs), duct/gland (D/G) cells, horizontal basal cells (HBCs), globose basal cells (GBCs), immature olfactory sensory neurons (OSNs), mature OSNs, and sustentacular (sus) cells (Supplementary Fig. 2). Samples were then screened for *Nrg1* using primers for *cEGF*, and qRT-PCR results were normalized to *Nrg1* in sus cells, a cell type negative for Nrg1 expression based on no detectable amplification after 40 rounds of PCR (Data not shown).

All populations show at least a 4-fold increase of *Nrg1* over sus cells, with the neuronal populations expressing close to 15-fold more *Nrg1* (Supplementary Fig. 3). Furthermore, a thorough analysis of these 7 populations using Nrg1 isoform-specific PCR primers reveals a cell type-specific expression pattern (Fig. 2). For these calculations, expression levels of isoform-specific *Nrg1* in each population were normalized to expression in the whole mucosa.

D/G cells express the highest levels of *Nrg1*, primarily Type I (5-fold) and α -TMC (8-fold) isoforms with a minor contribution of β -TMC (Fig. 2A). We previously showed that Type I is highly expressed in the lamina propria compared with epithelium, and this is likely due to the fact that the gland subpopulation is exclusive to the lamina propria. D/G cells are

followed in *Nrg1* expression by the mature neurons, which express Type I (3.5 fold) and uniquely express Type III isoforms (1.8 fold) (Fig. 2B). The relatively high abundance of neurons in comparison to other cell types contributes to the earlier observation of high Type III expression in the epithelium. HBCs primarily express the Type I α -TMC *Nrg1* isoform (2 fold) (Fig. 2C). Finally, OECs, which are in the lamina propria compartment have a slight preference for Type I (1.5 fold) and express both α and β -TMC (2 fold and 3.5 fold respectively) (Fig. 2D). Ultimately, these results indicate that the Type I isoform is expressed predominantly in the D/G population (and most likely in the gland subgroup) while Type III expression is present primarily in the mature OSN population (Fig. 2A,B). In addition, the presence of various EGF isoforms and the lack of any strong presence of the “a” or “b” termination exon in any population suggests that there does not seem to be a strong cell type-specific preference for these regions and shows that the highest cell type specificity is for 5' exon variance.

To test this hypothesis, we acquired antibodies that were specific to either Type I or Type III and looked for expression in the uninjured adult mouse olfactory mucosa. In agreement with the RNA level analysis above, *Nrg1* Type I shows strong protein level expression exclusively in the D/G cells, as confirmed by co-staining with the D/G marker Sox9 (Fig. 3A). In addition, *Nrg1* Type III shows strong protein level expression in mature OSNs, and complete absence from the immature OSNs (Fig. 3B,C).

2.3. EGFR, ErbB2, and ErbB3 are expressed in the olfactory mucosa

The ErbB receptors are the only known receptors for *Nrg1*. Although *Nrg1* can only bind and activate ErbB3 and ErbB4, these 2 receptors are capable of heterodimerization with EGFR and ErbB2 (Falls, 2003). Therefore, in order to begin to address the functional activity of *Nrg1*, we first sought to determine the expression pattern of all 4 ErbB receptors in the olfactory mucosa (Peles et al., 1993; Plowman et al., 1993; Carraway et al., 1994).

An antibody specific to ErbB3 shows expression in D/G cells (Fig. 4A) and not in OSNs or HBCs (Fig. 4B,C). In addition to D/G cells, ErbB3 also shows expression in a subpopulation of microvillar cells that are TrpM5+ (Fig. 4D–F). To date, the characterization and function of microvillar cells are still being fully defined, although they are thought to exist as a signaling cell mediating tissue homeostasis as well as being pheromone responsive (Pfister et al., 2012; Lopez et al., 2014; Yamaguchi et al., 2014).

ErbB4 expression in the olfactory system has previously been described only in the interneurons of the olfactory bulb and reported to be entirely absent from the olfactory epithelium (Perroteau et al., 1998; Vullhorst et al., 2009). Since ErbB3 is not capable of functional signaling as a homodimer, it must be present as a heterodimer with either EGFR or ErbB2.

EGFR has previously been shown to be expressed in both HBCs and sus cells (Holbrook et al., 1995). However, despite the presence of EGFR in the epithelium, it is not co-expressed with ErbB3 in HBCs and thus cannot participate as a heterodimerization partner for ErbB3.

The remaining potential partner for ErbB3 is ErbB2, however an antibody specific to ErbB2 shows strong expression in immature OSNs but not in mature OSNs or D/G cells (Fig. 5). To verify this expression, we looked specifically at the *Neurog1*(+) population of immature OSNs and progenitors using a *Neurog1-eGFP* mouse and found that ErbB2 is indeed expressed specifically in these cells (Fig. 5A–C). Since it appeared that a subset of ErbB2(+) cells were not *Neurog1*(+), but rather were situated more basally, we looked at neuronal cell lineage populations further upstream from immature OSNs and saw expression in all *Ascl1*/*Mash1*(+) and *NeuroD1*(+) GBCs (Fig. 5D,E). Moreover, ErbB2 expression is also found in the population of multipotent, *Pax6*(+) GBCs (Fig. 5F). ErbB2 is not present in the underlying HBC progenitor population (Fig. 5G). As suggested by its presence in GBCs, a subpopulation of ErbB2(+) cells is also actively dividing (Fig. 5H). Additionally, we confirmed that ErbB2 expression was restricted to these groups of immature cells by showing its absence in the differentiated populations of mature neurons and D/G cells (Fig. 5I–K).

To confirm that ErbB2 could not be a heterodimerization partner for ErbB3 in the uninjured epithelium, we looked for overlapping expression by histology. In the uninjured adult mouse, ErbB2 is not co-expressed with ErbB3 in the epithelium or with *Nrg1* Type I D/G cells in the lamina propria (Supplementary Fig. 4). This suggests that despite the presence of *Nrg1* and the ErbB receptors, lack of overlapping heterodimerization partners likely prevents *Nrg1* signaling through ErbB3 in the uninjured epithelium.

2.4. Altered ErbB expression following MeBr lesion

We next performed immunohistochemistry on MeBr-lesioned tissue from adult animals at various days post-lesion to analyze changes in *Nrg1* Type I, ErbB3, and ErbB2 patterns of expression. Interestingly, *Nrg1* Type I expression dramatically changes following MeBr-lesion, extending from the lamina propria into the epithelium by 2 dpl and persisting in the epithelium for at least 4 dpl. Severe lesions take longer to regenerate and extend this window of *Nrg1* Type I expression in the OE for up to 7 days when compared to a standard lesion (Fig. 6 E,F). *Nrg1* Type I expression remains largely absent from HBCs, a cell population activated upon MeBr-lesion (Fig. 6), and persists in D/G cells (Supplementary Fig. 5). Sparse labeling of cells positive for HBC markers is observed, but it is minimal and generally apical, suggesting it is in HBCs that are transitioning into a GBC state. These results correlate with the isoform summary showing an increase of *Nrg1* Type I in the epithelium after lesion (Fig. 1B). *Nrg1* Type III is absent during the early regenerative phase and appears exclusively in the mature neuronal population that does not appear until late in regeneration.

We then looked to see whether ErbB2/ErbB3 expression changed in such a way that these two could function as hetero-dimerization partners after injury. ErbB3 expression is highly increased from 2 dpl through 4 dpl, with expression seen in both D/G cells as well as other cell types (Fig. 7A–E). As the EGFR(+) HBCs activate and proliferate during this timeframe as an initial response to injury, we looked to see if ErbB3 was co-localized in HBCs. We found that ErbB3 expression in HBCs is present, but minimal, appearing to be restricted to the most apical, and likely transitioning HBCs (Fig. 7F–J) The other dimerization partner,

ErbB2, was previously shown to be expressed in populations of progenitor cells (Fig. 5), suggesting that it could possibly be expressed in many cells following lesion, including ErbB3(+) cells. To that extent, ErbB2 is upregulated at 4 dpl, and is found to be strongly overlapping with ErbB3 (Fig. 7K–O). Finally, Nrg1 Type I shows strong co-expression with ErbB3 from 2 dpl through 4 dpl, situating all three signaling partners in an overlapping expression pattern at 4 dpl (Fig. 7P–T).

3. Discussion

Our results demonstrate that Nrg1 is strongly expressed in the olfactory mucosa and that it is differentially regulated following MeBr injury. In-depth analysis of isoform expression revealed that of up to 42 putative and 15–20 confirmed isoforms, only 9–10 are present in the olfactory mucosa (Fig. 1 D, E). Of these 9–10 isoforms, all but one contain the functional domains required for downstream ErbB signaling. The remaining isoform represents a novel splice variant that is likely non-functional or a dominant negative due to the lack of a complete EGF domain (Type I-EGF-a) (Wen et al., 1994; Marikovsky et al., 1995; Baek and Kim, 1998). Additionally, for the β 3-EGF variant, we were not able to verify whether it was present with only one or with both Type I and Type III N-terminal domains. We propose that this should be further investigated, as β 3-EGF represents an early termination isoform lacking a C-terminus that contains crucial cell surface trafficking information (Liu et al., 1998a,b). As a result, its cell surface localization and therefore ability to participate in ErbB signaling can only occur when paired with the Type III N-terminal exon. When this early truncation is instead paired with the Type I N-terminal exon, this variant would remain in the cytosol (Marchionni et al., 1993). Some data suggest that rather than resulting in a non-functional protein, the cytosol-restricted Type I- β 3-EGF isoform is translocated to the nucleolus where it may participate in gene regulation through ribosome synthesis (Golding et al., 2004). Thus, in the right context all of the variants characterized here are capable of some type of bioactive function dictated by their exon composition.

3.1. Isoform variability

The N-terminal region of Nrg1 determines whether the protein is secreted (Type I) or membrane-bound (Type III). Our results show that both types of Nrg1 are present in the olfactory mucosa, supporting the idea that both paracrine and juxtacrine signaling modes are important for olfactory epithelium development, maintenance, and/or repair. In accordance with known tissue-specific localization patterns, Nrg1 Type III is found primarily in mature OSNs in the epithelium, while Type I is found in the mesenchyme (Yang et al., 1998; Wolpowitz et al., 2000).

Both Type I and Type III variants are found to contain overlapping sets of EGF-domain and C-terminal ICDs. While the choice of type directly alters the kind of signaling possible, i.e., secreted vs. membrane bound, the EGF variants alter signaling by changing the kinetics of processing.

β -EGF isoforms of Nrg1 exhibit increased binding and activation efficacy with ErbB receptors compared to α -EGF isoforms (Wen et al., 1994; Pinkas-Kramarski et al., 1996).

The ability to select between high and low affinity isoforms provides a regulatory mechanism that is common during development. Furthermore, it has been shown that the EGF domain of Nrg1 is capable of binding integrins to form a ternary structure with ErbB3 (Ieguchi et al., 2010). Subtle modifications in this region may add another level of regulation to the ternary complex formation kinetics. Further downstream biochemical analysis of phosphorylation for Nrg1 both *in vivo* and through the utilization of *in vitro* cell free systems would help to characterize a role for α - and β -EGF in the epithelium.

A third variation to the EGF domain seen in the olfactory mucosa is the presence or absence of a small (24 nucleotide) “stalk” region on the EGF domain carboxy-terminus. This stalk region is seen only with the β -EGF domain in Type I and Type III variants, and represents the most common EGF isoform seen in both the lamina propria and the epithelium (Fig. 2). The presence of the stalk ensures optimal proteolytic processing of the extracellular domain and release of either the Type I secreted product or exposure of the Type III membrane-bound ligand (Montero et al., 2000). Our finding that this is the predominant variant suggests that Nrg1 (both Type I and III) is efficiently processed into an active ligand throughout the olfactory mucosa. It should be noted that although we did find β -EGF to exist without the stalk region in the epithelium by qRT-PCR (Fig. 2B, pink), we were unable to clone this iso-form out using our TOPO® cloning approach (Fig. 2E,F). This is likely due to the fact that without highly saturating numbers, the TOPO® cloning approach is much less sensitive compared to qRT-PCR. Indeed, less than 10% of the total EGF isoforms lack the stalk region (Fig. 2B, pink).

The final region of isoform variance found in the epithelium, is in the C-terminal ICD, where the presence of both “a” and “b” was found in the lamina propria and the epithelium (Fig. 2). For Type I, the presence of either variant is necessary to traffic the receptor to the cell membrane since Type I does not contain a membrane-localization signal in its N-terminus (Liu et al., 1998a). Though the exact functional differences between the “a” and “b” variant are unknown, if they were equivalent, selection of the C-terminal ICD should be unbiased toward either variant. However, our data show that Type I exists only with the “a” tail in the epithelium, suggesting an additional and unknown role for the tail region that would lead to preferential selection (Supplementary Fig. 1). Unlike Type I, Type III contains a membrane-localizing signal, and thus the C-terminal ICD offers the unique possibility for bidirectional signaling with the “a” variant. In this scenario, the ICD can act as an inducible transcription factor upon translocation to the nucleus where it regulates genes responsible for neuron survival and maintenance (Bao et al., 2003; Bao et al., 2004). This scenario is highly relevant in the epithelium, where Type III Nrg1 is abundant in mature OSNs (Fig. 5B). Indeed, mRNA analysis shows that selection of the “a” variant occurs in 61% of the spliced isoforms in uninjured epithelium, suggesting that the “a” variant that allows bidirectional signaling is preferred.

In addition to the “a” and “b” ICD, we did find minimal presence of the “c” ICD. This is an early truncation form, lacking the C-terminal membrane-localization signal. As the Type I variants require this signal, the “c” ICD containing isoforms would result in nonfunctional or dominant negative protein. Although the “c” ICD would result in functional protein when combined with the Type III exon, it comprises only 5% (lamina propria) and 4%

(epithelium) of the spliced isoforms (Fig. 2A,B, red), below the detection limit for TOPO® analysis (10%), and not likely to have an important role in signaling.

While this pattern of exons and splice variants in uninjured tissue suggests a role for low-level tissue maintenance of cellular function, the olfactory mucosa is capable of significant amounts of epitheliopoiesis after injury. We therefore investigated whether these exon preferences of Nrg1 would be dynamic during regenerative conditions. Indeed, after lesion, Type I expression shifts to the epithelium and persists from 2 dpl up to 7 dpl (Fig. 7P–T). Not only does expression shift to the epithelium, but it also increases in the lamina propria, resulting in an overall upregulation of Nrg1 Type I in the mucosa. In addition, the extent of Type I expression is directly proportional to lesion severity (Fig. 6E,F). Interestingly, while the type variation is fairly straightforward, changes in the EGF domain are complex, with epithelium and lamina propria biases changing in opposite directions after lesion. Early changes in expression from 2 dpl suggest that these regulatory differences are occurring in progenitor populations.

3.2. Differential expression of Nrg1 and ErbB receptors in olfactory mucosa

We show that Nrg1 Type I is expressed in D/G cells in the lamina propria (Fig. 3A), a cell type that has an unexplored progenitor capacity and is capable of giving rise to at least D/G and sus cells following epithelial lesion (Schwob et al., 1995; Huard et al., 1998). Although the qRT-PCR analysis of FACS isolated cell populations suggests that Type I and Type III are expressed in more cell types than what is observed using these isoform-specific antibodies, it is important to remember that the sensitivity of RT-qPCR is far higher than IHC, and that RNA abundance does not always correlate with protein expression. In addition, antibodies that can distinguish between EGF and C-terminal variants do not currently exist. However, based on the RNA data from samples post-MeBr (Fig. 1), we are able to make a few inferences.

First, the increase of α -EGF in the epithelium (and decrease in the lamina propria) likely reflects the expansion/proliferation of D/G cells in the epithelium after lesion, and suggests that this variant may be important to the injury response. An increase in the α -EGF isoform has previously been seen in the olfactory epithelium following injury and further confirmation of this finding suggests that the α -EGF variant may be of particular importance during regeneration (Williams et al., 2004). Second, the increase in β 1-EGF in the lamina propria after injury may reflect a need for increased paracrine signaling through more efficient proteolytic release of secreted Nrg1. Third, the increase of the “b” ICD in the epithelium after injury could support the idea that most Type III present in mature OSNs contains the “a” ICD, providing a capacity for bidirectional signaling, whereas MeBr-induced neuronal death leads to a decrease in “a” and a resultant increase in the “b” ICD. These hypotheses could be further tested by looking at both uninjured and MeBr-injured tissue, using isoform-specific RNA probes designed for *in situ* hybridization.

As with the Type I isoform, all ErbB receptors expressed in the mucosa (EGFR, ErbB2, and ErbB3) are expressed in cell types with progenitor capabilities (HBCs, GBCs, and D/G cells, respectively). Importantly, we identified a cell-type specific expression pattern for ErbB2 in all precursor cells of GBC neuronal lineage that was previously unknown and characterized

the expression of ErbB3 in D/G cells of both the lamina propria and the epithelium (Salehi-Ashtiani and Farbman, 1996). Despite the proximity of ErbB3 and Nrg1 Type I in D/G cells, without a heterodimerization partner, ErbB3 is not able to participate in Type I-induced signaling. Indeed, the differential expression patterns of the ErbB receptors actually prevent the possibility for heterodimerization and therefore ErbB signaling in any cell prior to injury.

After injury, on the other hand, not only do ErbB2 and ErbB3 show an upregulation in expression, but more importantly, by 4 dpl they are both co-expressed in a subset of cells in the epithelium (Fig. 7D,I,N,S). ErbB3 also shows some localization with CK14 at both 3 and 4 dpl in a population that is apical to the EGFR(+) HBCs, however this population likely consists of HBCs transitioning to a GBC state and shedding their HBC identity, making functional ErbB3 signaling in HBCs improbable. Finally, the combined upregulation of ErbB2, ErbB3, and Nrg1 Type I provide a permissive environment within which Nrg1-induced ErbB signaling could occur.

4. Conclusion

These results meticulously outline the expression of Nrg1 iso-forms and ErbB receptors throughout the olfactory mucosa in uninjured as well as MeBr-injured, regenerating conditions. Due to the highly segregated expression patterns of both the ligand Nrg1 and its cognate ErbB receptors, we show that uninjured tissue contains no Nrg1-mediated ErbB signaling. Following injury, however, the regenerating tissue alters expression to bring the ligand and receptor into close proximity to allow for functional Nrg1 signaling. This level and type of regulation present in such a highly regenerative tissue as the mucosa suggests that the multitude of Nrg1 isoforms should have a critical and functional importance in an environment of regeneration. Taken more broadly, our results propose and enable further studies in examining the role of Nrg1 in not only olfactory mucosa, but also other tissues with neurogenic capacity.

5. Experimental procedures

5.1. Animals and breeding

Wildtype F1 mice used for MeBr-lesion, tissue harvest for RNA analysis, and all IHC (unless otherwise indicated), were bred from C57/BJ6 and 129S1/Sv1MKJ mice in-house or ordered from Jackson Labs (#101043). *Sox2egfp* mice have been previously described, and are an ES-cell knock-in mouse strain (Ellis et al., 2004). These mice generate GFP in all cell types that pass through a Sox2-expressing stage, including globose basal cells (GBCs), horizontal basal cells (HBCs), and sustentacular (sus) cells. *Neurog1eGFP* and *Hes5::GFP* mice have previously been described by the GENSAT project and are BAC transgenic lines (Gong et al., 2003; Tomishima et al., 2007). These mice produce GFP in immature olfactory sensory neurons (*Neurog1eGFP*) and OECs (*Hes5::GFP*) of the olfactory epithelium. Conventional transgenic K5creER^{T2} mice have been described elsewhere (Indra et al., 1999) and were provided by P. Chambon and R. Reed. K5creER^{T2} mice were crossed with a cre-inducible Tdtomato mouse from Jackson Laboratory (# 007909) to produce a K5creER^{T2}:Tdtomato reporter line, which expresses Tdtomato in HBCs after tamoxifen

administration. *OMP-eGFP* have previously been described, and were provided by P. Mombaerts (Potter et al., 2001). These mice were produced by insertion of GFP into the OMP locus and result in GFP expression in mature olfactory sensory neurons (OSNs) of the epithelium.

5.2. MeBr lesion

12 week old C57/BJ6.129 (F1) mice were exposed to 175 ppm MeBr diluted in air at 10 L/min for 8 h as previously described (Schwob et al., 1995). Unilateral lesions were performed by inserting a plug consisting of a 5 mm piece of PE10 tubing occluded by a knotted 7.0 suture into the right naris of the mouse, and sealing the naris with superglue.

5.3. Drug administration

Tamoxifen (Sigma) was prepared as a 30 mg/mL stock in corn oil and administered to mice at 4 weeks of age. K5creER^{T2}:Tdtomato mice were IP-injected with 275 mg/kg and sacrificed 2 weeks post-injection.

5.4. Tissue harvesting

Mice were anesthetized with a lethal dose of the triple cocktail of ketamine (37.5 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.25 mg/kg) via IP injection. Following anesthetization, mice were transcardially flushed with 10 mL cold PBS.

For RNA analysis—Following PBS flushing, mice were perfused with 10 mL Low Ca²⁺ Ringer Solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM EDTA, 10 mM glucose, 1 mM sodium pyruvate, pH7.2). The turbinates were then removed from the septum and discarded. The olfactory mucosa consists of epithelium sitting on a basal lamina proximal to the deeper mesenchymal tissue of the lamina propria. These were separated as follows. For unlesioned mice, starting at the dorsal region, the epithelium was peeled away from the septum leaving the lamina propria attached to the bone. Separated tissue was flash frozen on dry ice. Tissue from 5 animals was combined for each replicate.

For lesioned mice, animals were euthanized at 2 days post-lesion and subjected to *en bloc* proteolytic digestion in order to dissociate epithelium cells while leaving the lamina propria relatively undisturbed. The septum was incubated for 5 min in 0.05% Trypsin-EDTA in a 37 °C waterbath. Solution was drained off and replaced with an enzyme cocktail containing (100 U/mL collagenase, 250 U/mL hyaluronidase, 75 U/mL DNase I, 0.1 mg/mL trypsin inhibitor, 5 U/mL papain; from Worthington Biochemical, Roche, and Sigma) for 30 min at 37 °C with light agitation every 10 min. Tissue was then filtered through 120 µm mesh to separate the epithelium (filtrate) from the lamina propria (unfiltered tissue). The lamina propria was washed 1× with Regular Ringer Solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM EDTA, 10 mM glucose, 1 mM sodium pyruvate, 1 mM CaCl₂, 1 mM MgCl₂, pH7.2) and then flash frozen on dry ice. The epithelium was washed 1× with Regular Ringer Solution and filtered through a 35 µm mesh before flash freezing. Tissue from 3 animals was combined for each replicate.

For FACS analysis—Following PBS flushing, mice were perfused with 10 mL Low Ca^{2+} Ringer Solution. After dissection, the septum and turbinates were finely minced and incubated for 5 min in 0.05% Trypsin-EDTA in a 37 °C waterbath (for lamina propria fibroblasts, OECs, HBCs, immature OSNs, and mature OSN respective mouse strains). The solution was drained off and replaced with an enzyme cocktail containing (100 U/mL collagenase, 250 U/mL hyaluronidase, 75 U/mL DNase I, 0.1 mg/mL trypsin inhibitor, 5 U/mL papain; from Worthington Biochemical, Roche, and Sigma) for 30 min at 37 °C with light agitation every 10 min. Tissue was then filtered through 120 μm mesh followed by 35 μm mesh before being washed in Staining Solution (25 mM HEPES, 10 mM EDTA in 1 $\times$ HBSS). For duct/gland (D/G) cells, GBCs, and sus cells, *Sox2egfp* mouse tissue was dissociated identically, except for 2 changes: 1) after mincing, the cells are placed directly in the enzyme cocktail, and 2) the enzyme cocktail is slightly changed (500 U/mL collagenase, 250 U/mL hyaluronidase, 75 U/mL DNase I, 5 U/mL papain; from Worthington Biochemical, Roche, and Sigma).

For IHC analysis—Following PBS flushing, mice were perfused with 1% PLP (1% PFA, 0.01 M monobasic and dibasic phosphates, 90 mM lysine, 0.1 M sodium periodate). After dissection, tissue was post-fixed for 1 h in 1% PLP under vacuum pressure and decalcified overnight in saturated EDTA. Tissue was then cryoprotected in 30% sucrose in PBS, embedded in OCT (Miles Inc., Elkhart, IN), and frozen in liquid nitrogen.

5.5. RNA isolation, RT-qPCR, and TOPO sequencing

RNA was extracted using TRI Reagent® (Ambion) according to the manufacturers instructions. To generate cDNA, 500 ng of RNA was reverse-transcribed using SuperScript III RT (Invitrogen). Primers for qPCR are listed in Table 1A, and all samples were amplified using SYBR® Green RT-PCR Master Mix (Ambion). Primers for PCR and gel electrophoresis of single-splice variants of *Nrg1* are listed in Table 1B. To determine full-length *Nrg1* isoforms, primers listed in Table 1C were used to amplify *Nrg1* from epithelial and lamina propria samples, and the entire PCR product was cloned into the TOPO® vector (Invitrogen). The M13R primer was used to sequence clones.

5.6. FACS

All staining of lamina propria fibroblasts, D/G cells, GBCs, and sus cells, was done in suspension in 2 mL glass conical tubes with antibody dilutions prepared in Staining Solution (25 mM HEPES, 10 mM EDTA in 1 $\times$ HBSS). Primary antibody incubation was for 20 min on ice with mixing by pipetting every 10 min. Secondary and tertiary antibodies were added for 10 min each. Cells were washed between incubations with Staining Solution. Antibodies used are listed in Table 2. Each biological replicate contains pooled mouse samples based on target cell type abundance (OEC-8 mice; lamina propria fibroblast-5 mice; D/G/GBC/sus-5 mice; HBC-3-5 mice; immature OSNs-4 mice; mature OSNs-2 mice).

5.7. Immunohistochemistry

Primary antibodies and their respective protocols are listed in Table 3. Prior to immunostaining, tissue sections were rinsed in PBS and subjected to one of multiple pretreatments: A) ethanol dehydration/rehydration involved 1 min incubations in the

following sequence of ethanol baths: 50%, 90%, 100%, 90%, 50%, B) steaming slides in 0.01 M citrate buffer (pH6.0) in a commercial steamer for 5 or 10 min, C) steaming slides in 0.01 M citrate buffer (pH6.0) at 60 °C for 30 min, D) DNase treating slides at room temperature for 10 min in 500 U/mL DNase I in DNase I Buffer (40 mM Tris–HCl, 10 mM NaCl, 6 mM MgCl₂, 10 mM CaCl₂, pH7.9), E) 5 min incubation in 3% H₂O₂ in MeOH, F) 5 min incubation in MeOH. Following all pretreatments, slides were rinsed in PBS and blocked for 1 h at room temperature in Normal Donkey Block (10% donkey serum, 5% non-fat dry milk, 4% BSA, 0.1% TritonX-100; in PBS). Slides were incubated in primary antibody overnight and on the second day were visualized using a variety of techniques described in Table 3. Tissue was imaged on a Nikon 800E epifluorescence microscope using a SPOT RT2 digital camera. All images were prepared in Photoshop CS2. Only levels and evenness of illumination were adjusted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Baek SY, Kim SU. Proliferation of human Schwann cells induced by neu differentiation factor isoforms. *Dev Neurosci*. 1998; 20(6):512–517. [PubMed: 9858839]
- Bao J, Lin H, et al. Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. *Nat Neurosci*. 2004; 7(11):1250–1258. [PubMed: 15494726]
- Bao J, Wolpowitz D, et al. Back signaling by the Nrg-1 intracellular domain. *J cell Biol*. 2003; 161(6): 1133–1141. [PubMed: 12821646]
- Britsch S, Li L, et al. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes & Dev*. 1998; 12(12):1825–1836. [PubMed: 9637684]
- Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr Opin Neurobiol*. 2001; 11(3):287–296. [PubMed: 11399426]
- Burgess T, Ross S, et al. Biosynthetic processing of neu differentiation factor. Glycosylation trafficking, and regulated cleavage from the cell surface. *J Biol Chem*. 1995; 270(32):19188–19196. [PubMed: 7642587]
- Carraway KL 3rd, Sliwkowski MX, et al. The erbB3 gene product is a receptor for heregulin. *J Biol Chem*. 1994; 269(19):14303–14306. [PubMed: 8188716]
- Dong Z, Brennan A, et al. Neu differentiation factor is a neuroglia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron*. 1995; 15(3):585–596. [PubMed: 7546738]
- Ellis P, Fagan BM, et al. SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. *Dev Neurosci*. 2004; 26(2–4):148–165. [PubMed: 15711057]
- Falls DL. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res*. 2003; 284(1):14–30. [http://dx.doi.org/10.1016/S0014-4827\(02\)00102-7](http://dx.doi.org/10.1016/S0014-4827(02)00102-7). [PubMed: 12648463]

- Golding M, Ruhrberg C, et al. Mapping nucleolar and spliceosomal localization sequences of neuregulin1-beta3. *Exp Cell Res*. 2004; 299(1):110–118. [PubMed: 15302578]
- Garratt A, Britsch S, et al. Neuregulin, a factor with many functions in the life of a schwann cell. *BioEssays News Rev Mol Cell Dev Biol*. 2000; 22(11):987–996.
- Gong S, Zheng C, et al. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature*. 2003; 425(6961):917–925. [PubMed: 14586460]
- Holbrook EH, Szumowski KE, et al. An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. *J Comp Neurol*. 1995; 363(1):129–146. [PubMed: 8682932]
- Holmes W, Sliwkowski M, et al. Identification of heregulin, a specific activator of p185erbB2. *Sci (New York, NY)*. 1992; 256(5060):1205–1210.
- Huard JMT, Youngentob SL, et al. Olfactory epithelium contains multi-potent progenitors that give rise to neurons and non-neuronal cells. *J Comp Neurol*. 1998; 400(4):469–486. [PubMed: 9786409]
- Ieguchi K, Fujita M, et al. Direct binding of the EGF-like domain of neuregulin-1 to integrins ({alpha}v{beta}3 and {alpha}6{beta}4) is involved in neuregulin-1/ErbB signaling. *J Biol Chem*. 2010; 285(41):31388–31398. [PubMed: 20682778]
- Indra AK, Warot X, et al. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res*. 1999; 27(22):4324–4327. [PubMed: 10536138]
- Kramer R, Bucay N, et al. Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc Natl Acad Sci U S A*. 1996; 93(10):4833–4838. [PubMed: 8643489]
- Li L, Cleary S, et al. The breast proto-oncogene, HRGalpha regulates epithelial proliferation and lobuloalveolar development in the mouse mammary gland. *Oncogene*. 2002; 21(32):4900–4907. [PubMed: 12118369]
- Liu X, Hwang H, et al. Domain-specific gene disruption reveals critical regulation of neuregulin signaling by its cytoplasmic tail. *Proc Natl Acad Sci U S A*. 1998a; 95(22):13024–13029. [PubMed: 9789034]
- Liu X, Hwang H, et al. Release of the neuregulin functional polypeptide requires its cytoplasmic tail. *J Biol Chem*. 1998b; 273(51):34335–34340. [PubMed: 9852099]
- Lopez F, Delgado R, et al. Transduction for pheromones in the main olfactory epithelium is mediated by the Ca2+-activated channel TRPM5. *J Neurosci off J Soc Neurosci*. 2014; 34(9):3268–3278.
- Marchionni MA, Goodearl AD, et al. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature*. 1993; 362(6418):312–318. [PubMed: 8096067]
- Marikovsky M, Lavi S, et al. ErbB-3 mediates differential mitogenic effects of NDF/heregulin isoforms on mouse keratinocytes. *Oncogene*. 1995; 10(7):1403–1411. [PubMed: 7731691]
- Meyer D, Birchmeier C. Multiple essential functions of neuregulin in development. *Nature*. 1995; 378(6555):386–390. [PubMed: 7477375]
- Meyer D, Yamaai T, et al. Isoform-specific expression and function of neuregulin. *Dev Camb Engl*. 1997; 124(18):3575–3586.
- Montero J, Yuste L, et al. Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor-alpha-converting enzyme. *Mol Cell Neurosci*. 2000; 16(5):631–648. [PubMed: 11083924]
- Peles E, Ben-Levy R, et al. Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. *EMBO J*. 1993; 12(3):961–971. [PubMed: 8096177]
- Peles E, Yarden Y. Neu and its ligands: from an oncogene to neural factors. *BioEssays News Rev Mol Cell Dev Biol*. 1993; 15(12):815–824.
- Perroteau I, Oberto M, et al. ErbB-3 and ErbB-4 expression in the mouse olfactory system. *Ann N Y Acad Sci*. 1998; 855:255–259. [PubMed: 9929617]
- Pfister S, Dietrich MG, et al. Characterization and turnover of CD73/IP(3) R3-positive microvillar cells in the adult mouse olfactory epithelium. *Chem senses*. 2012; 37(9):859–868. [PubMed: 22952298]

- Pinkas-Kramarski R, Shelly M, et al. Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. *J Biol Chem*. 1996; 271(32):19029–19032. [PubMed: 8702572]
- Plowman GD, Green JM, et al. Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. *Nature*. 1993; 366(6454):473–475. [PubMed: 7902537]
- Potter S, Zheng C, et al. Structure and emergence of specific olfactory glomeruli in the mouse. *J Neurosci off J Soc Neurosci*. 2001; 21(24):9713–9723.
- Salehi-Ashtiani K, Farbman AI. Expression of neu and Neu differentiation factor in the olfactory mucosa of rat. *Int J Dev Neurosci off J Int Soc Dev Neurosci*. 1996; 14(7–8):801–811.
- Schwob JE, Youngentob SL, et al. Reconstitution of the rat olfactory epithelium after methyl bromide-induced lesion. *J Comp Neurol*. 1995; 359(1):15–37. <http://dx.doi.org/10.1002/cne.903590103>. [PubMed: 8557844]
- Shah N, Marchionni M, et al. Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell*. 1994; 77(3):349–360. [PubMed: 7910115]
- Tomishima M, Hadjantonakis AK, et al. Production of green fluorescent protein transgenic embryonic stem cells using the GENSAT bacterial artificial chromosome library. *Stem cells Dayt Ohio*. 2007; 25(1):39–45.
- Vullhorst D, Neddens J, et al. Selective expression of ErbB4 in interneurons, but not pyramidal cells, of the rodent hippocampus. *J Neurosci off J Soc Neurosci*. 2009; 29(39):12255–12264.
- Wadugu B, Kuhn B. The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. *Am J physiol Heart circ physiol*. 2012; 302(11):H2139–H2147. [PubMed: 22427524]
- Wang J, Miller S, et al. The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. *J Biol Chem*. 2001; 276(4):2841–2851. [PubMed: 11042203]
- Wen D, Peles E, et al. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell*. 1992; 69(3):559–572. [PubMed: 1349853]
- Wen D, Suggs SV, et al. Structural and functional aspects of the multiplicity of Neu differentiation factors. *Mol Cell Biol*. 1994; 14(3):1909–1919. [PubMed: 7509448]
- Williams, Sarah K.; Franklin, RJM.; Barnett, Susan C. Response of olfactory ensheathing cells to the degeneration and regeneration of the peripheral olfactory system and the involvement of the neuregulins. *J Comp Neurol*. 2004; 470(1):50–62. <http://dx.doi.org/10.1002/cne.11045>. [PubMed: 14755525]
- Wolpowitz D, Mason T, et al. Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. *Neuron*. 2000; 25(1):79–91. [PubMed: 10707974]
- Yamaguchi T, Yamashita J, et al. Skn-1a/Pou2f3 is required for the generation of Trpm5-expressing microvillous cells in the mouse main olfactory epithelium. *BMC Neurosci*. 2014; 15:13. [PubMed: 24428937]
- Yang X, Kuo Y, et al. A cysteine-rich isoform of neuregulin controls the level of expression of neuronal nicotinic receptor channels during synaptogenesis. *Neuron*. 1998; 20(2):255–270. [PubMed: 9491987]

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.gep.2015.10.001>.

Fig. 1. Olfactory epithelium and lamina propria have differing expression preferences of Nrg1 isoforms, and react to injury differently

(A) Schematic outlining the known exons that are differentially spliced in the Nrg1 gene. For clarity, a consistent color scheme was used for the exons which are part of the three main variable regions (N terminal, EGF, and C terminal). (B,C) Each variable exon is clustered in their respective domain and the expression levels in the olfactory epithelium (B) or lamina propria (C) are depicted as a percentage of total Nrg1 (n = 3 for each tissue type). Black bars represent the levels expressed during uninjured normal tissue, while white bars represent levels in tissue two days post injury from inhaled methyl bromide gas. (SEM error bars, T-test, *p < 0.05, **p < 0.001) (D,E) The full-length isoform composition and percentage of total Nrg1 in olfactory epithelium (D) or lamina propria (E) is depicted as a nested pie chart. The size of each slice is representative of the relative amount of the labeled exon. Contiguous fragments moving from the center to outside represents a full length isoform. For example, in the olfactory epithelium, a small (6.8%) of total Nrg1 is Type I-α-a. For clarity in the figure, the EGF variable domain is placed on the larger, outside ring.

Fig. 2. Expression of variable exons of Nrg1 in FACS-purified cell type-specific populations qRT-PCR results of RNA extracted from (A) Duct/Gland cells, (B) Mature Neurons, (C) HBCs, (D) OECs, (E) Immature Neurons, (F) GBCs, (G), LP Fibroblasts. CT values each exon were normalized to β -actin, averaged, and plotted as relative to whole mucosa (WM). (SEM error bars, $n = 3$ (all except immature neurons, $n = 7$)).

Fig. 3. Spatial Expression Pattern of Nrg1 Type I and Type III in the normal olfactory mucosa (A) An antibody against Nrg1 Type I shows expression in Sox9(+) duct/gland cells in the lamina propria. (B) An antibody against Nrg1 Type III shows expression in OMP(+) mature OSNs, and (C) absence from Tuj(+) immature OSNs. Arrows indicate Type I (+) Duct/Gland cells, while arrowheads indicate location of the basal lamina. Insets highlight regions of co-localization. Nuclei are visualized with DAPI. Scale bar = 50 μ m.

Fig. 4. Spatial expression pattern of Nrg1 Receptor ErbB3 in the normal olfactory mucosa (A) An antibody against ErbB3 shows expression in Sox9(+) duct/gland cells and (B) absence from PGP9.5(+) OSNs and (C) CK14(+) HBCs. (D–F) ErbB3 also labels TrpM5(+) microvillar cells on the apical layer of the epithelium. Arrows indicate co-localization. Insets highlight regions of co-localization. Nuclei are visualized with DAPI. Scale bar = 20 μ m.

Fig. 5. Spatial expression pattern of Nrg1 Receptor ErbB2 in the normal olfactory mucosa (A–C) Co-staining of ErbB2 receptor with a Neurog1-eGFP reporter, a marker for downstream neural progenitors. (D–G) Co-staining of ErbB2 with the more upstream progenitor markers *Ascl1* (*Mash1*), *NeuroD1*, *Pax6*, and *CD54*, respectively. (H,I) ErbB2 co-stains with the proliferative marker *Ki67* in addition to overlapping with the immature neuronal marker *NCAM*. (J,K) ErbB2 does not colocalize with the mature neuronal marker *OMP* or the duct/gland marker *ECAD*. Nuclei are visualized with DAPI. Scale bar = 20 μ m.

Fig. 6. Nrg1 Type I expression in the olfactory mucosa following MeBr lesion

(A) Nrg1 Type I expression at 1–4 days post lesion (dpl), showing restriction from CD54(+) HBCs and (B–C) increased expression in the olfactory epithelium. (E) Areas of the mucosa that are subjected to a harsher lesion have increased levels of Nrg1 Type I present in the epithelium at 7 dpl compared to (F) epithelium that has had a less harsh lesion. Arrows indicate Nrg1 Type I expression in the epithelium. Nuclei are visualized with DAPI. Scale bar = 20 μ m.

Fig. 7. Dynamics of ErbB3 expression in the olfactory mucosa after MeBr lesion

(A) ErbB3 staining shows expression in Sox9(+) duct/gland cells in the lamina propria 1 dpl. (B,C) ErbB3 expression is seen in duct/gland cells at 2–3 dpl, and is enhanced in non-duct/gland cells by 4 dpl. (E) ErbB3 is again expressed primarily in duct/gland cells by 7 dpl. (F,G) ErbB3 is not expressed in CK14(+) HBCs at 1–2 dpl, but (H,I) appears co-localized with a small, apically-situated CK14(+) population at 3–4 dpl. (J) by 7 dpl, ErbB3 is again restricted from all CK14(+) HBCs. (K–M) ErbB2 is not detected at 1–3 dpl, (N) but at 4 dpl, ErbB2 and ErbB3 are co-expressed. (O) By 7 dpl, ErbB2 and ErbB3 are again mutually exclusive. (P) Nrg1 Type I and ErbB3 are expressed in different populations of cells in the lamina propria at 1 dpl, but (Q–S) from 2–4 dpl, they are co-localized in both lamina propria and epithelial populations of duct/gland cells. (T) At 7 dpl, Nrg1 Type I expression is once again restricted from the OE and no longer co-localizes with ErbB3. Insets highlight regions of co-localization. Nuclei are visualized with DAPI. Scale bar = 20 μ m.

Table 1

Primers used in this study.

Isoform	Forward	Reverse
A. Primers for Nrgl Isoform-Specific OCR		
Type I	5'-TGA GCG CAA AGA AGG CAG AGG CA-3'	5'-CAA TGC TGG GCT CGG GTC GC-3'
Type II	5'-GCC CAC TCA GCC TTC CCC TC-3'	5'-GCC AAT GCG CAC CGC TT-3'
Type III	5'-ACT TTC CAC ATC TAC ATC CAC GA-3'	5'-TCT TGA GGG CTT TGA CAG GTC C-3'
α1-EGF	5'-CAC GAC TGG GAC CAG CCA TCT C-3'	5'-CCC AAG ATG CTT TTC TTG GGT TTG-3'
α-EGF	5'-CCA AAC CM AGA AAA AGC GGA GGA-3'	5'-GGC CAC CAC ACA CAT GAT GCC G-3'
β1-EGF	5'-GAC TGG CAC CAG CCA TCT CAT-3'	5'-GAT GCT TGT AGA AGC TGG CCA TTA-3'
β3-EGF	5'-GTG CAA GTC CCC AAA TGA CTT-3'	5'-CCT GGT AGA AGG CTC TAG CTC-3'
βEGF	5'-GGC CAG CTT CIA CAA AGC GGA GGA-3'	5'-CCC ACC ACA CAC ATG ATG CCG AC-3'
γ-EGF	5'-GGA CCA GCC ATC TCA TM A-3'	5'-TTA CTT GCA TTG CAC AAG TAT-3'
a ICD	5'-AGT CAA ACG TAT GTA TCA GCC ATG A-3'	5'-GCG TCA CAA GAA GCA GAG GC-3'
b ICD	5'-CAT CGG TAG AGA ACA GCA GGC-3'	5'-AGG TTA TGC TIT CAC TAT GAG GAG A-3'
c ICD	5'-TGA GCA CCT GCG GTC TCT CCT-3'	5'-TGG ACA GCT GGC CGA TIC GA-3'
cEGF	5'-ACG ACT GGG ACC AGC CAT CTC AT-3'	5'-ACG GGT TTG ACA GGT CCT TCA CCA T-3'
β Actin	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAACAATGCCATGT-3'
B. Primers for Nrg1 PCR and Gel Electrophoresis of Single-Splice Variants		
Type I- β	5'-CCG AGC CCA GCA ITC CTT CC-3'	5'-TCA ITT GGG ACT TGC ACA ACT ATC-3'
Type I- α	5'-GAG CCC AGC ATT GCC TCC CA-3'	5'-TCC AGG TGG GCA CTT GCA CAA GT-3'
Type III-β	S'-GCT GCC TCC GCT GTT CTG GTC T-3'	5'-TCA TIT GGG CAC TTG CAC AAG TAT C-3'
C. Primers used to Amplify Full-Length Nrgl for TOPO Cloning		
Ia	5'-TGA CCC CAA AGA AGG CAG AGG CA-3'	5'-AGG GGG CGA TIT AGG GGA GCT T-3'
Ib	5-TGA GCG CAA AGA AGG GAG AGG CA-3'	5'-TGC ATG CAT TTG GAT LTG TAA GCC T-3'
IIIa	5'-CCC AAG TCA GGA ACT CAG CCA CA-3'	5'-AGG GGG CGA ITT AGG GGA GCT T-3'
IIIb	5-CCC AAG TCA GGA ACT CAG CCA CA-3'	5'-TGC ATG CAT TIG GAT CTG TAA GCC T-3'
Primer		Reverse
D. Sequencing Primer used with TOPO Cloning		
M13R		5-CAG GAA ACA GCT ATG AC-3'

Table 2

Antibodies used for FACS.

Primary antibody	Vendor	Protocol	Specificity
Thy1.2	eBiosciences	(1:100)->bDaRat (1:200)->SA-488 (1:500)	LP Fibroblast
CD54	R&D Systems	(1:100)->bDaG (1:200)->SA-647 (1:600)	HBCs
ECAD	Sigma	(1:1500)->bDaRat (1:200)->SA-647 (1:600)	D/G and Sus

Table 3

Antibodies used for IHC.

Primary antibody	Source/Vendor	Pretreatment	Protocol	Specificity
GtαCD54	R&D Systems	3% H ₂ O ₂ + EtOH	(1:100)-Fluor-Gt	HBCs
RbαCK14	Lab Vision	3% H ₂ O ₂ + 5 min Steam	(1:150)->Fluor-Rb	HBCs
RtαECAD	Sigma	3% H ₂ O ₂ + 10 min steam	(1:200)->bDaRt->SA-HRP->FITC TSA	D/G and Sus
ShpαEGFR	Abcam	1. 3% H ₂ O ₂ + 10 min steam 2. 3% H ₂ O ₂ + EtOH 3. 3% H ₂ O ₂ + 5 min steam	(1:30)->Fluor-Rb (with NCAM,ECAD) (1:75)->Fluor-Rb (with Pax6) (1:200)->Fluor-Rb (with Ki67, GFP) (1:400)->Fluor-Rb (with Mash1, ND1, OMP) (1:400)->Fluor-Rb (with CD54) (1:20)->Fluor-Rb (with ErbB3)	D/G
ShpαErbB3	R&D Systems	1. 3% H ₂ O ₂ + 5 min steam 2. Citrate Buffer 3. 10 min steam + MeOH	(1:5)->bDaShp->fluor-SA (with ErbB2, Sox9, PGP9.5, CK14) (1:25)0 > TxRedαShp->ShpαTxRed->594-Shp (with Trpm5) (1:10)0 > bDaShp->fluor-SA (with Nrg1 Type I)	D/G
MaErbB4	Millipore	Dnase + Meoh + 10 min steam	(1:250)->TxRedαM->MaTxRed->594-M	Interneurons of the olfactory bulb
Gt-αGFP	Abcam	3% H ₂ O ₂ + 10 min steam	(1:80)->488-Gt	GFP
MaKi67	BD Bioscience	3% H ₂ O ₂ + 10 min steam	(1:100)0 > FITCaM->MaFITC->488-M	Dividing cells
MaMash1	BD Bioscience	3% H ₂ O ₂ + 10 min steam	(1:125)->bDaM->SA-HRP->FITC-TSA	GBC-TA
RtαNCAM	Abcam	3% H ₂ O ₂ + 10 min steam	(1:20)->FITCaRt->RtαFITC->488-Rt	Immature OSN
GtαNeuroD1	Santa Cruz	3% H ₂ O ₂ + 10 min steam	(1:1000)->bDaGt->SA-HRP->FITC-TSA	GBC-NC
RbαNrg1 Type I	Abcam	1. 10 min steam 2. 10 min steam + MeOH	(1:25)->Fluor-Rb (with Sox9) (1:25)->Fluor-Rb (with ErbB3)	D/G
RbαNrg1 Type III	Abcam	1. 3% H ₂ O ₂ 2. EtOH	(1:5)->TxRedαRb->RbαTxRed->594-Rb (with OMP) (1:10)->TxRedαR->RbαTxRed->594-Rb (with Tuj)	Mature OSN
GtαOMP	Wako	1. 3% H ₂ O ₂ 2. 3% H ₂ O ₂ + EtOH	(1:120)->fluor-Gt (with Nrg1 Type III) (1:120)->fluor-Gt (with ErbB2)	Mature OSN
RbαPax6	Chemicon	3% H ₂ O ₂ + 10 min steam	(1:6)->bDaRb->SA-HRP->FITC-TSA	GBC-MPP
RbαPGP9.5	Ultracelone	3% H ₂ O ₂ + 5 min steam	(1:1200)->fluor-Rb	All OSNs
Rb αSox9	Millipore	1. 10 min steam 2. 3% H ₂ O ₂ +5 min steam	(1:10,000)->bDaRb->SA-HRP->FITC-TSA (with Nrg1 Type I) (1:500)->fluor-Rb (with ErbB3)	D/G and Sus
GPαTrpM5	Gift from E. Liman (USC,CA)	Citrate	(1:300)->bDaGP->fluor-SA	Microvillar cell
MaTuj1	Covance	EtOH	(1:500)->fluor-M	All OSNs