Developmental Regulation of Gonadotropin-releasing Hormone Gene Expression by the MSX and DLX Homeodomain Protein Families[*]

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Abstract

Gonadotropin-releasing hormone (GnRH) is the central regulator of the hypothalamic-pituitary-gonadal axis, controlling sexual maturation and fertility in diverse species from fish to humans. *GnRH* gene expression is limited to a discrete population of neurons that migrate through the nasal region into the hypothalamus during embryonic development. The *GnRH* regulatory region contains four conserved homeodomain binding sites (ATTA) that are essential for basal promoter activity and cell-specific expression of the *GnRH* gene. MSX and DLX are members of the Antennapedia class of non-Hox homeodomain transcription factors that regulate gene expression and influence development of the craniofacial structures and anterior forebrain. Here, we report that expression patterns of the *Msx* and *Dlx* families of homeodomain transcription factors largely coincide with the migratory route of GnRH neurons and co-express with GnRH in neurons during embryonic development. In addition, MSX and DLX family members bind directly to the ATTA consensus sequences and regulate transcriptional activity of the *GnRH* promoter. Finally, mice lacking MSX1 or DLX1 and 2 show altered numbers of *GnRH*-expressing cells in regions where these factors likely function. These findings strongly support a role for MSX and DLX in contributing to spatiotemporal regulation of *GnRH* transcription during development.

Proper sexual maturation and fertility are dependent upon the correct function of the hypothalamic-pituitary-gonadal axis, initiated by a small, yet critical population of

gonadotropin-releasing hormone (GnRH)[1] neurons. The *GnRH* gene is expressed in a complex spatiotemporal manner during embryonic development and into postnatal life with several populations of *GnRH*-expressing neurons originating at different developmental stages and locations. These populations include the classical, septohypothalamic neurons, as well as populations in the lateral septum, posterior bed nucleus stria terminalis (pBNST), and tectum (1,2). Although the role of each of these populations of *GnRH*-producing neurons remains to be elucidated, the contribution of the septohypothalamic population is required for maturation of the hypothalamic-pituitary-gonadal axis and fertility (3).

The precursor cells of the septohypothalamic GnRH neurons have been reported to originate within the olfactory placode (4–6) or the neural crest (7) and begin to express the *GnRH* transcript in a discrete population of cells located in close proximity to the olfactory placode of the embryonic mouse by 11.5-days postcoitum (11.5 dpc). By 12.5 dpc, the full complement of septohypothalamic GnRH neurons (~800) in the adult population is established (6), and over the course of the next several days, these neurons migrate toward the CNS, closely associated with the established position of the peripherin-positive nerve bundle of the olfactory nerve (8,9). By 16.5 dpc the majority of the septohypothalamic neurons have reached their destination, scattered throughout the preoptic area, the diagonal band of Broca and the medial septum (5,10). Expression of GnRH during prenatal development actively contributes to the identity and migratory ability of the septohypothalamic population. Indeed, GnRH itself, is involved in furthering the commitment and migratory state of the GnRH neuronal precursor in an ultrashort, positive feedback loop by upregulating expression of the *GnRH* gene, stimulating axon growth, cytoskeletal remodeling and increasing the migration of neuronal precursors (11). Thus, expression of GnRH during embryonic development likely promotes the characteristics of the maturing GnRH neuron that are essential for reproductive ability.

Because of the limited number and dispersed nature of GnRH neurons, the difficulty in examining *GnRH* promoter expression *in vivo* is evident. The generation of immortalized GnRH neuronal cell lines, the GT1-7, GN11, and NLT cells, has provided cellular model systems in which to study GnRH (12,13). Whereas the murine hypothalamic cell line, GT1-7, exhibits key characteristics of a mature, fully differentiated *GnRH*-expressing neuron, the GN11 and NLT cell lines are migratory in culture and suggested to represent immature GnRH neurons (12–15). Studies with these two models have been invaluable for identifying the protein transcription factor complexes that facilitate expression of *GnRH*.

Both *in vitro* and *in vivo* studies have demonstrated that well defined promoter and enhancer regions of the *GnRH* gene, containing ATTA homeodomain-binding sites, are both necessary and sufficient for proper *GnRH* gene expression during embryonic development and in the adult (12,16). Moreover, these homeodomain-binding sites are essential for appropriate basal, as well as cell-specific expression of the *GnRH* promoter (17,18). A role for homeoproteins, such as MSX and DLX, in regulation of gene expression during development is well documented in vertebrate and invertebrate species (19–23). Interestingly, the MSX and DLX homeodomain transcription factor families have the appropriate expression patterns in the developing olfactory region and fore-brain of the mouse to be considered as potential regulators of the *GnRH* gene (24,25). These factors can also occupy homeodomain binding sites in a variety of upstream promoter regions (26–28). Here, we report the contribution of DLX and MSX family members to regulation of *GnRH* promoter expression *in vivo* and *in vitro*. We show that spatiotemporal expression of Msx and Dlx family members coincides with the migratory, septohypothalamic GnRH neuronal population. MSX and DLX family members are also expressed in the GnRH model cell lines, bind to upstream regulatory regions of the

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[1]The abbreviations used are: GnRH, gonadotropin-releasing hormone; dpc, days postcoitum; EMSA, electromobility shift assay; DIG, digoxigenin; CNS, central nervous system.

GnRH gene, and regulate promoter activity *in vitro*. In addition, analyses of mouse mutants revealed that DLX1 and -2 as well as MSX1 contribute to proper *GnRH* expression in the prenatal septohypothalamic areas. Therefore, we propose that MSX and DLX family members participate in developmental regulation of *GnRH* gene expression.

MATERIALS AND METHODS

Nuclear Extract Preparation and Electromobility Shift Assays (EMSA)

Nuclear extracts for EMSA were prepared according to Clark and Mellon (29). Oligonucleotide probes were annealed and labeled with [γ-^{32}P]ATP (6000 Ci/mmol; PerkinElmer Life Science Products) in a polynucleotide kinase reaction. Radiolabeled probes were then cleaned over MicroSpin G-25 columns (Amersham Biosciences). 1 fmol of probe was incubated with 2 μg from GT1-7 cells or 6 μg from NLT or GN11 cells of nuclear protein extract in binding buffer (50 mM KCl, 10 mM HEPES (pH 7.8), 1 mM EDTA, 5 mM spermidine, 2% Ficoll, 1.25 mM dithiothreitol, 6% glycerol, and 4 mM phenylmethylsulfonyl fluoride) for 5 min before loading on a 5% polyacrylamide nondenaturing gel. Gels were run at 250 V for 2 h, then dried under a vacuum and exposed to film for 1–3 days. For supershift assays, 1 or 2 μl of anti-DLX1 (Chemicon AB5724), DLX2 (Chemicon AB5726), DLX5 (Chemicon AB5728), MSX1 (Covance MMS-261R), or MSX2 (Santa Cruz SC-15396) antibody was incubated in the reaction mix for 5 min before loading.

Cell Culture and Transfection

GT1-7, GN11, and NLT cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 4.5% glucose in a 5% CO_2 atmosphere at 37 °C. Cells were split into 24-well plates for transfection. 400 ng of pGL3-*GnRH*e/p reporter or pGL3-mutant *GnRH*e/p reporter were co-transfected with 100 or 200 ng of pCB6+-Msx1, pCMV-Msx2, pCDNA3-Dlx5, pCAGGS-Dlx1, or pCB6+-Dlx2 expression vectors in addition to 200 ng of thymidine kinase-β-galactosidase reporter vector as an internal control using FuGENE 6 reagent (Roche Applied Science). The mutant *GnRH*e/p reporter contained a linker-scanner mutation deleting the two homeodomain enhancer sites (−1636 to −1631 and −1623 to −1618) (17) and two point mutations in each of the promoter sites, −55 to −50 and −42 to −38 (CAATTA to CAgTTg and CATTA to CgTTg). Transfections were harvested 24 h later in lysis buffer (100 mM potassium phosphate, pH 7.8; 0.2% Triton X-100) to yield cellular proteins. β-Galactosidase assays were performed as advised by the manufacturer (Tropix, Bedford, MA), and luciferase assays were performed as previously described (17). To control for transfection efficiency, luciferase values were normalized to β-galactosidase activity. Values of the normalized expression vectors were compared with empty vector control or an RSVe/RSVp control. All experiments were performed in triplicate and repeated a minimum of three times.

In Situ Hybridization and Immunohistochemistry

Mouse colonies were maintained in agreement with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. The *Dlx1&2*-null, or *Msx1*-null embryos were produced by heterozygous intercrosses of *Dlx1&2* (30) or *Msx1*-nLacZ (31), and maintained in a C57BL/6J background. Fertilization was assumed to occur at midnight, and pregnant females were sacrificed at 12–16 dpc accordingly to harvest embryos (noon on the day after finding the plug is considered 0.5 dpc). Genotypes were confirmed by PCR as previously described (30,31).

Mouse embryos were fixed (10% acetic acid, 30% formaldehyde, 60% ethanol) overnight at 4 °C and then dehydrated in a series of ethanol/water washes before embedding in paraffin. Sections were cut ~7-μm thick and then placed onto Superfrost Plus Slides (Fisher) and left at

room temperature to dry overnight. Sections were then deparaffinized with xylene washes, hydrated in ethanol/water solutions and digested with 1 μg/ml proteinase K for 7 min at 37 °C, followed by postfixation in 10% neutral buffered formalin for 20 min at room temperature. The sections were then washed with 1× phosphate-buffered saline and 2× SSC for 5 min.

Antisense probes corresponded to sequences as previously reported for *Dlx2* (25), *Msx1* and *Msx2* (32), *Dlx1* and *Dlx5* (33,34). The *GnRH* antisense sequence corresponds to 325 bp of the cDNA from +1 to the endogenous BamHI site. Probes corresponding to the sense sequence were used as controls. Briefly, 1 μg of linearized plasmid DNA was incubated with 10× digoxigenin (DIG) RNA labeling mix (Roche Applied Science) as well as 5× transcription buffer (Promega) and an RNA polymerase T7, T3, or SP6 for 2 h at 37 °C. The reaction was stopped by adding 0.2 M EDTA, pH 8.0, and probes were then cleaned over G-50 MicroSpin columns (Amersham Biosciences).

The DIG-labeled probes, diluted in hybridization buffer (1× NaCl, 50% formamide, 10% dextran sulfate, 1 mg/ml yeast RNA, 1× Denhardt's solution) were added to slides and incubated overnight at 65 °C. Sections were washed three times at 65 °C in 50% formamide, 1× SSC, with 0.1% Tween-20 and then twice at room temperature in MABT (100 mM maleic acid, pH 7.5, 150 mM NaCl, 0.1% Tween-20). The slides were blocked with MABT plus 5% normal goat serum for 3–4 h. The hybridized DIG-labeled probe was detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) at a dilution of 1:2000 and visualized with the chromogen combination 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT). The single probe *in situ* hybridization sections were counterstained with nuclear fast red (Vector).

Immunohistochemical analysis was performed after *in situ* hybridization. These slides were soaked in immunogen stabilizing buffer (1 mM citric acid, 8 mM sodium citrate) for 20 min at 65 °C to retrieve the antigen. Immunohistochemistry was then carried out as previously described (16). The GnRH peptide was recognized with the LR-1 antibody at a dilution of 1:2000 (gift of R. Benoit) and visualized using the horseradish peroxidase-conjugated ABC kit (Vector).

Counting of the GnRH-positive neurons was carried out by sectioning whole embryo heads (starting from the inner ear upon appearance of the cochlear partition) in sagittal orientation at ~10-μm thickness and performing *in situ* hybridization on all sections as described above to identify *GnRH*-expressing cells. All sections were tallied to obtain the number of GnRH-positive cells. Approximately 150 sections were analyzed per embryo head for the earlier developmental stages, and 250 sections were analyzed per embryo head for the later developmental stage. Three or more embryos were examined at each time point with the exception of the *Dlx1&2* embryos analyzed at 16.5 dpc, which represent two whole embryo heads.

Reverse Transcriptase PCR

Total RNA extraction was carried out as described by the method of Chomczynski and Sacchi (35). The reverse transcriptase reaction was performed according to the methods of Pernasetti *et al.* (36). PCR analysis was done with 30 cycles of: 45 s at 95 °C, 45 s at the proper annealing temperature for the primers, and 1 min at 72 °C, followed by an elongation step for 10 min at 72 °C. Specific primers for *Msx1-3*, *Dlx1-6*, and *β*-actin were used in the PCR reaction. PCR products were separated by electrophoresis on a 2% agarose gel.

RESULTS

Msx and Dlx Are Candidates for Regulating GnRH during Embryonic Development

Although ongoing research has examined the human and mouse *GnRH* gene promoters, the rat gene has been investigated most extensively. The two well characterized regulatory regions of the rat *GnRH* gene are a 300-bp enhancer located at −1863 to −1571 relative to the start site and an evolutionarily conserved 173-bp promoter just 5′ of the transcriptional start site. Within these regulatory regions, four conserved, consensus homeodomain sites (ATTA) have been identified (Fig. 1, *panel A*) (17,18). These sites were extensively characterized and shown to be essential for appropriate basal and cell-specific expression of the *GnRH* promoter (17,18). Thus, identifying the transcriptional regulators that occupy and function through these homeodomain sites is important for understanding the regulation of GnRH transcriptional activity.

Several candidate homeoproteins were considered for their binding properties, developmental expression patterns, and transcriptional activity, among them the MSX and DLX families of transcriptional regulators. Given the previously described similarities in expression patterns of these transcription factors and the septohypothalamic population of GnRH neurons (24,25), we sought to compare the expression patterns of *Dlx1*, *Dlx2*, *Dlx5*, *Msx1*, and *Msx2* to that of *GnRH* in the embryonic forebrain and nasal region during development. Adjacent series of parasagittal sections were taken from mouse embryos at age 13.5 dpc, when the septohypothalamic population of GnRH neurons has been established. By RNA *in situ* hybridization, we observed a trail of *GnRH*-expressing neurons localizing from the olfactory placode, along the olfactory nerve and crossing the cribriform plate into the anterior forebrain, caudal to the olfactory bulb, in a region termed the primordium of the septum (Fig. 1, *panels B*, *E*, and *H*). Adjacent sections show strong expression of *Msx1* and *2* (*panels G* and *I*, respectively) as well as *Dlx1*, *2*, and *5* (*panels C*, *D*, and *F*, respectively) in the nasal region and anterior forebrain, consistent with their previously reported expression patterns. These results indicate that the spatial expression of these *Msx* and *Dlx* family members correlates with the developing septohypothalamic GnRH neuronal population.

We then examined the expression of Msx and Dlx family members in the model GnRH neuronal lines, GT1-7 and NLT/GN11. Reverse transcriptase PCR was performed on RNA from the GT1-7, GN11, NLT, and NIH3T3 cell lines, as well as total olfactory bulb RNA to assess the *Msx* and *Dlx* family members being expressed. *Msx1*, *2*, and *3*, as well as *Dlx2*, *4*, *5*, and *6*, were expressed in the GT1-7 cells (Fig. 2*A*). Interestingly, NLT cells expressed *Msx2* and *3* but not *Msx1*, and *Dlx1* but not *Dlx2* through *6*. This same expression profile was seen in GN11 cells (data not shown). As expected, based on previous observations *in vivo*, *Dlx* family members were expressed in the olfactory bulb (37). In addition, the NIH3T3 cells served as a negative control for expression of *Msx1* or *Dlx2* (17). Thus, distinct members of the *Dlx* and *Msx* families are expressed in the different GnRH model cell lines.

Consistent with the RT-PCR studies *in vitro*, we found that both *Msx* and *Dlx* family members co-expressed with GnRH-positive neurons at embryonic stages. Using RNA probes for the *Msx* and *Dlx* family members and an antibody recognizing the GnRH peptide, we performed double *in situ* hybridization/immunohistochemistry on sagittal sections of 13.5-dpc embryos. Fig. 2*B* shows expression of *Msx1* in the olfactory epithelium and along the olfactory sensory neuron tracts in the nasal region and GnRH-immunoreactive neurons expressing *Msx1* in this region (Fig. 2*B*, *expanded panel*). *Msx2* exhibited a similar expression pattern to that of *Msx1* and neurons co-expressing *Msx2* and GnRH along the olfactory sensory neuron tracts in the nasal region were observed (Fig. 2*C*, *expanded panel*). Fig. 2*D* indicates GnRH neurons located along the cribriform plate and in the forebrain primordium of the septum that expressed *Dlx1* (expanded panel). *Dlx2* exhibited a similar expression pattern to that of *Dlx1* and also

was co-expressed with GnRH-positive neurons (Fig. 2*E*, *expanded panel*). Like *Dlx1* and *2*, *Dlx5* was expressed in GnRH-positive cells in the forebrain. However, we also observed GnRH neurons in the nasal region expressing *Dlx5* (data not shown). These results show that *Msx* and *Dlx* are expressed in the GnRH-positive neurons during embryonic development and are plausible candidates for regulating *GnRH* promoter activity through the conserved homeodomain binding sites.

DLX and MSX Bind to Conserved ATTA Sequences in the GnRH Enhancer and Promoter

Previously, detailed analyses of the protein complexes binding to the ATTA homeodomain sites in the upstream region of the GnRH gene suggested that they are composed of Q50 homeodomain proteins (17,18). MSX and DLX are members of the Antennapedia class of non-Hox homeodomain proteins that contain a glutamine residue at amino acid position 50 (Gln-50) of the homeodomain (38). Furthermore, MSX and DLX have been shown to mediate transcription by binding consensus ATTA sites in upstream regulatory regions (28). Thus, we hypothesized that one or more members of the MSX and DLX families may regulate *GnRH* transcription by binding to ATTA sequences within the promoter and enhancer.

To determine whether these proteins could directly bind to the ATTA sequences in the *GnRH* enhancer and promoter, we performed EMSA with antibody supershifts (Fig. 3). Radiolabeled oligonucleotides containing the homeodomain sites of the *GnRH* enhancer and promoter were incubated with nuclear extracts from GT1-7 cells. As described previously (17,18), a specific complex was formed in GT1-7 nuclear extracts incubated with probes containing the ATTA sequences of the GnRH enhancer or promoter. Interestingly, a faint complex of faster mobility than the major complex also formed. This minor complex had been observed previously but not characterized (17,18). The addition of an antibody specific to MSX1 inhibited protein binding of the major complex (*thick arrow*). In contrast, an antibody against DLX2 supershifted the minor complex (*thin arrow*) on the probe representing the 5′-enhancer site, −1642/−1623 (Fig. 3*A*, *lanes 3* and *4*) as well as the complex formed on the promoter element (data not shown). These results indicate that the complexes formed in GT1-7 cells on the ATTA sites within the *GnRH* enhancer and promoter contain DLX2 and MSX1.

The EMSA binding pattern with the GT1-7 nuclear proteins on the DNA probe corresponding to the *GnRH* enhancer homeodomain binding site (−1642/−1623) is unique in comparison to many other patterns observed from non-*GnRH* producing cell lines (17,18). Interestingly, the binding pattern of GN11 and NLT cell nuclear extracts is similar to the GT1-7 cell nuclear extracts (17,18). Therefore, we explored the possibility that these similar complexes formed in GN11 and NLT cells might also contain DLX and MSX. Fig. 3*B* represents EMSA analysis of the homeodomain site in the *GnRH* enhancer incubated with NLT nuclear extracts. Five specific complexes formed on the *GnRH* enhancer probe with NLT extracts. Addition of an antibody recognizing DLX1 specifically inhibited binding of complex 4 (*thin arrow*) in NLT cells (Fig. 3*B*, *lane 2*), while an anti-MSX2 antibody inhibited binding of complex 3 (*thick arrow*, *lane 3*). EMSA performed with GN11 extracts showed the same binding pattern as that observed with the NLT extracts including results utilizing the anti-DLX1, or MSX2 antibodies (data not shown). Antibodies recognizing additional MSX and DLX family members did not supershift complexes formed on the −1642 probe (anti-MSX2 in the GT1-7 extract and anti-DLX5 in the GT1-7 and GN11/NLT extracts; data not shown). These results suggest that despite the molecular differences between the GT1-7 and GN11/NLT cells, the complexes bound to the homeodomain sites in the *GnRH* gene contain MSX and DLX family members in both model GnRH neuron cell lines.

DLX and MSX Regulate GnRH Gene Expression

Transcriptional assays have revealed that the MSX and DLX proteins interact with each other and recognize the same DNA consensus sequence (28). In addition, members of the MSX family have been characterized as transcriptional repressors, whereas the DLX family activates transcription (28,34,39–42). To characterize the effect of these transcription factors on the expression of the *GnRH* gene, we expressed MSX or DLX family members in the GT1-7 cells by transient co-transfections. The *GnRH* reporter plasmid utilized consists of the well characterized regulatory regions of the *GnRH* gene, the evolutionarily conserved enhancer and promoter (*GnRH*e/p), driving the luciferase gene. These DNA sequences are sufficient to target appropriate expression to GnRH neurons *in vivo* (16). MSX1 and MSX2 repressed *GnRH*e/p reporter activity to 47 and 36%, respectively, as compared with an empty vector control (Fig. 4). In contrast, expression of DLX2 or DLX5 in the GT1-7 cells increased activity of the *GnRH*e/p reporter 169 and 254%, respectively, while introduction of DLX1 increased reporter activity 227%. Moreover, co-transfection of expression vectors for DLX1, 2, and 5 together significantly increased reporter activity (417%) over the empty vector control. These results show that MSX expression represses, while DLX expression activates *GnRH* transcription in GT1-7 cells.

Having identified four homeodomain sites in the *GnRH* promoter that are bound by MSX and DLX *in vitro*, we next asked whether the activities of the MSX and DLX family members were dependent upon these ATTA sequences. Mutations previously shown to inhibit protein binding to the homeodomain element (17,18) were introduced in the *GnRH*e/p-luciferase vector at the two enhancer sites (−1636 to −1631 and −1623 to −1618) and the two promoter sites (−55 to −50 and −42 to −38). Interestingly, transient transfection of the reporter plasmid containing these four mutated sites (mutant *GnRH*e/p) in GT1-7 cells revealed 70% reduced reporter activity compared with a wild-type *GnRH*e/p reporter (Fig. 4*B*), demonstrating that these sites are important for proper basal activity of the *GnRH* promoter in GT1-7 cells. Although the mutant *GnRH*e/p reporter showed greatly reduced basal reporter activity compared with the wild-type plasmid, activity was still significantly above background. While expression of MSX1 or MSX2 significantly reduced activity of the *GnRH*e/p reporter, it did not significantly affect the mutant *GnRH*e/p reporter. Further, expression of DLX2 or DLX5 activated the *GnRH*e/p reporter but showed no statistically significant increase in activity on the mutant *GnRH*e/p reporter. However, co-expression of DLX1 in concert with either the wild-type or mutant *GnRH*e/p reporter significantly increased transcriptional activity, although activation was reduced with the mutant reporter. These results suggest that MSX and DLX family members function through the homeodomain binding sites and these sites are important for *GnRH* promoter activity.

MSX and DLX Compete for Occupation of the Q50 Homeodomain Binding Sites and Show Functional Antagonism on the GnRH Gene

Given the opposing transcriptional activity of MSX and DLX family members on the *GnRH* promoter (Fig. 4), we asked whether the major (MSX) and minor (DLX) complexes compete for binding to the ATTA elements of the *GnRH* regulatory region. Competition between MSX1 and DLX2 for DNA binding has been previously described and indicates that while MSX1 and DLX2 can form heterodimers, this interaction is mutually exclusive of DNA binding (28). To address this question, we generated nuclear extracts from GT1-7 cells overexpressing the DLX2 protein for EMSA analysis. As seen in Fig. 5*A*, the intensity of the minor complex increased while the major complex had reduced intensity compared with the binding pattern of nontransfected GT1-7 nuclear extract (Fig. 5*A*, *lanes 2* and *1*, respectively). These same results were seen in the extracts from NLT cells overexpressing the DLX1 protein (Fig. 5*B*). Furthermore, in transient transfection of GT1-7 cells, expression of increasing amounts of DLX2 overcame MSX1-repressed *GnRH* promoter activity (Fig. 5*C*). These results indicate

that MSX1 and DLX2 compete for occupation of the ATTA repeats in the *GnRH* enhancer and exhibit functionally antagonistic activity.

Differential GnRH Promoter Expression in Model GnRH Neuronal Cell Lines

Characterization of the GT1-7 and GN11 or NLT cell lines suggests that the GT1-7 cells represent a differentiated, mature GnRH neuron while the GN11 or NLT cells mimic the immature, migratory stages of GnRH neuron development (12–15). Previous findings suggest that GnRH expression itself is involved in furthering the commitment and migratory state of the GnRH neurons (11). We therefore hypothesized that *GnRH* transcriptional activity would differ between the migratory and mature GnRH neuronal cell lines. We examined the level of *GnRH* promoter activity in these cells by transient transfection with the *GnRH*e/p reporter. Because the GN11 cell line has higher transfection efficiency than the NLT line, these cells were used to investigate reporter activity. *GnRH* promoter activity in the GT1-7 cells was more than 11-fold higher than a pGL3-luciferase control (Fig. 6*A*). Interestingly, GN11 cells transiently transfected with the *GnRH*e/p reporter showed a greater than 20-fold reduction in promoter activity compared with the GT1-7 cells despite the lack of a statistical difference in expression of the control plasmid. Finally, the *GnRH* regulatory regions showed extremely low levels of activity in the NIH3T3 cells (Fig. 6*A*), as the activity of the *GnRH*e/p reporter in these cells was significantly lower than that of an empty pGL3-luciferase vector, suggesting that the promoter may be actively repressed in these cells.

One explanation for the significantly reduced activity of the *GnRH*e/p in GN11 cells may be the absence of key transcriptional activators in the GN11 cells that are present in the GT1-7 cell line. Indeed, using RT-PCR (Fig. 2*A*), we did not detect expression of *Dlx2-6* in the GN11/ NLT cells. Thus, we explored the possibility that expression of the DLX family members in GN11 cells would restore the level of *GnRH* transcription in these cells. Transient co-transfection of DLX1, 2 and 5 with the *GnRH*e/p reporter in the GN11 cells significantly increased *GnRH* promoter expression by 13-fold (Fig. 6*B*). Furthermore, DLX activation of *GnRH* promoter expression required the presence of the homeodomain binding sites within the *GnRH* enhancer and promoter. This suggests that the reduced promoter activity of the *GnRH* gene in the GN11 cells may be caused by the absence of the DLX family of activators.

MSX1 and DLX1 and 2 Contribute to the Development of the GnRH Neuronal Population in Vivo

The data we have presented herein indicate that DLX1 and 2 function as key regulatory activators and MSX1 as a repressor of *GnRH* promoter expression through binding to homeodomain elements within the GnRH regulatory region (Figs. 3–5). In addition, double *in situ* hybridization/immunohistochemical analysis revealed co-expression of *Msx* and *Dlx* in the developing GnRH-positive neurons (Fig. 2). To determine whether MSX1 or DLX1 and DLX2 play a role in maintaining proper spatial and temporal *GnRH* expression *in vivo*, we performed RNA *in situ* hybridization to compare the number of GnRH-positive neurons between wild-type and *Dlx1&2*-null or *Msx1*-null mice at developmental stages when the complete population of septohypothalamic GnRH neurons has been established.

In the *Dlx1&2* background on 13.5 dpc, wild-type embryos had ~945 ± 62 GnRH-positive cells within the olfactory epithelium as well as along the cribriform plate and into the CNS, consistent with previous findings (6). In contrast, *Dlx1&2*-null littermates showed significantly reduced numbers of GnRH-positive neurons, ~612 ± 177 (Table I). Interestingly, the number of GnRH-positive neurons that have not yet crossed into the central nervous system was similar in the *Dlx1&2* wild-type (668 ± 74) and null (515 ± 125) mice. In contrast, the number of GnRH-positive neurons in the CNS of *Dlx1&2*-null mice (97 ± 60) was significantly reduced compared with *Dlx1&2* wild-type mice (277 ± 92) (Table I and Fig. 7, *panel A*). By 16.5 dpc,

Dlx1&2-null embryos had a similar number of septohypothalamic, GnRH-positive neurons overall. However, the position of these neurons had changed such that the majority of GnRH-positive cells were in the CNS. In the *Dlx1&2*-null mice there were 681 ± 81, whereas in the wild-type littermates there were 985 ± 16 total GnRH-positive neurons (Table I and Fig. 7, *panel B*). Thus, embryos lacking DLX1 and 2 have a reduced number of GnRH-positive cells in the septohypothalamic population, suggesting that DLX1 and DLX2 promote expression of *GnRH in vivo*.

In addition to the septohypothalamic population, another population of GnRH-positive cells was present at 16.5 dpc in the tectal region of the developing midbrain. Recent studies suggest that the tectal population is regulated by the same promoter elements as the septohypothalamic population but different transcription factor complexes because of the unique spatiotemporal expression of *GnRH* in the midbrain (2). We examined this population of GnRH-positive cells in the *Dlx1&2*-null embryos in order to assess *GnRH* promoter activity that is not directly regulated by DLX, as the *Dlx* genes are not expressed in this region of the brain. Interestingly, by *in situ* hybridization, we observed no differences between wild-type and null embryos in GnRH-positive cells in the tectal population (Table I and Fig. 7, *panels I* and *J*). This suggests that the reduced population of septohypothalamic, GnRH neurons in the *Dlx1&2*-null may be because of a loss of DLX1 and 2 and not an indirect, global, developmental misregulation.

Examination of the *Msx1* wild-type and null littermates at 12.5 and 13.5 dpc also revealed significant differences in the population of GnRH-positive cells. By 12.5 dpc, ~858 ± 18 GnRH-positive cells were present in the wild-type embryos while 1,032 ± 54 positive cells were observed in the *Msx1*-null littermates (Table I, Fig. 7, *panel C*). Although the difference did not reach statistical significance, the higher number of GnRH-positive cells seen in the null embryos could be attributed to a larger population of positive cells in the nasal region in addition to a few GnRH-positive cells in the olfactory epithelium (Fig. 7, *panel C*, *arrow*). Wild-type *Msx1* embryos on 13.5 dpc had ~939 ± 20 GnRH-positive cells scattered throughout the nasal region, extending into the primordium of the septum, while the *Msx1*-null embryos had significantly more (1,300 ± 116 and Table I). Interestingly, on 13.5 dpc, the nasal populations of GnRH-positive cells in *Msx1*-null embryos were significantly greater than those in wild-type littermates (1,021 ± 99 compared with 710 ± 44) (Table I and Fig. 7, *panel D*). Moreover, the GnRH-positive cells in the *Msx1*-null embryos were scattered throughout the nasal region but also located in the olfactory epithelium, outside of the typical location of the septohypothalamic population (Fig. 7, *panel F*, *arrows*). In addition, these cells were identified by an antibody recognizing the GnRH peptide, confirming that they indeed expressed GnRH (Fig. 7, *panel G*). There was also a small population of GnRH-positive cells (~20) located in the developing tectum at 13.5 dpc (Table I and Fig. 7, *panel H*), several days before these cells begin expressing *GnRH* in the wild-type embryos.

By 16.5 dpc, no significant difference in the number of GnRH-positive cells between wild-type and *Msx1*-null was observed. Approximately 871 ± 53 GnRH-positive cells were present in the *Msx1*-null embryos compared with 1,037 ± 103 in the wild-type embryos (Table I and Fig. 7, *panel E*). Interestingly, the nasal and CNS population of GnRH-positive cells did not differ between the wild-type and null embryos. Furthermore, the population of GnRH-positive cells located in the olfactory epithelium was still present at 16.5 dpc (Fig. 7, *panel E*, *arrows*). These findings suggest that *Msx1*, as an essential factor in the development of craniofacial morphology, also disrupts the development of the septohypothalamic population of GnRH neurons and likely regulates *GnRH* promoter expression during early stages of embryonic development.

In addition to the septohypothalamic population of GnRH-positive cells on 16.5 dpc, the tectal population of *GnRH*-expressing cells was present in the wild-type and *Msx1*-null embryos.

Tectal GnRH-positive cells could be seen in the *Msx1*-nulls as early as 13.5 dpc, though this population did not appear to differ in number or morphology in the 16.5-dpc embryos as compared with the wild-type litter mates (Table I and Fig. 7, *panel K*). Although the functional role for these tectal *GnRH*-expressing cells has not yet been elucidated, tectal *GnRH* expression is driven by the same promoter that regulates the septohypothalamic population (2) and, intriguingly, is also affected by an absence of MSX1 but not DLX1 and 2. The absence of MSX1, which is normally expressed in this region (43,44), may have resulted in premature differentiation or improper regulation of the *GnRH* promoter in these cells.

DISCUSSION

Regulated transcriptional activity forms the foundation for promoting the molecular identity and maturation of the septohypothalamic population of GnRH neurons during embryonic development. Investigation of immature, migratory and adult *GnRH*-expressing neurons has revealed differences in the expression pattern of transcriptional factors that regulate spatiotemporal transcriptional control of the *GnRH* gene (45,46). Thus, GnRH neurons are a heterogeneous population that differentially expresses specific genes at distinct stages of embryonic development, which may, in turn, result in dynamic regulation of *GnRH* promoter expression. Indeed, the transcriptional activity of the *GnRH* gene appears to be highly dynamic during the developmental, migratory stage of the septohypothalamic neurons. A recent study reported that the *GnRH* promoter expresses only at very low levels in neurons located in the olfactory region and becomes increasingly active in neurons in close proximity to the cribriform plate and in those that have entered the CNS (47). Thus, it has been reported that *GnRH* promoter activity is dependent upon the position of the neuron (47), which in turn is determined by the stage of embryonic development.

The findings presented herein implicate members of the DLX and MSX Q50 homeodomain families as important transcriptional regulators of *GnRH* gene expression. Repression and activation of the *GnRH* regulatory region by MSX and DLX, respectively, are dependent upon the conserved ATTA homeodomain sites (Fig. 4). Moreover, MSX and DLX bind identical homeodomain elements within the *GnRH* gene enhancer and promoter (Figs. 1 and 4*A*) and have functionally antagonistic activities (Fig. 5*B*). Interestingly, similar observations have been made in other contexts, such as the reciprocal activity of MSX2 and DLX5 on the osteocalcin (*OC*) promoter (48) and between PAX3 and MSX1 on the *MyoD* promoter (49).

In addition, we found that expression of *Msx* and *Dlx* family members coincides with the migratory route of septohypothalamic GnRH neurons during embryonic development (Fig. 2, *B–E*). The repressors, *Msx1* and *2* are expressed in the nasal region where *GnRH* promoter activity is low, while the transcriptional activators *Dlx1* and *2* are expressed at later times along the migratory route of GnRH neurons into the hypothalamus when *GnRH* promoter activity is high (47). In support of this, two model cell lines with characteristics of GnRH neurons at distinct developmental stages differ in their expression of several *Msx* and *Dlx* family members. The more mature GnRH neuronal cell model, GT1-7, expresses nearly all of the *Dlx* family of transcriptional activators as well as the *Msx* repressors (Fig. 2*A*). In contrast, the GN11/NLT cell lines, which represent the immature stage of the GnRH neuron, predominantly express the *Msx* repressors. Furthermore, *GnRH* promoter activity was 20-fold lower in GN11 *versus* GT1-7 cells, which may be due in part to absent expression of some DLX family members. Indeed, exogenous expression of the DLX family members in GN11 cells resulted in significant increases in *GnRH* promoter expression (Fig. 6*B*). Thus, we hypothesize that the coordinated spatial and temporal expression of *Msx* and *Dlx* family members in the embryo contributes to dynamic regulation of the *GnRH* promoter.

In support of our hypothesis, DLX and MSX family members appear to be required to maintain normal spatiotemporal populations of GnRH neurons in mice. Analysis of *Dlx1&2* and *Msx1*-null mutant mice revealed significant differences in the number of *GnRH*-positive neurons during development compared with wild-type mice (Table I). The reduced number of *GnRH*-positive cells in these *Dlx1&2*-null mutants may be directly due to misregulation of *GnRH* promoter activity. Whereas our *in vitro* and *in vivo* data strongly support the roles for DLX1 and DLX2 in *GnRH* expression, we cannot rule out an additional phenotypic contribution from decreased *Dlx5* expression, which has been observed in the *Dlx1&2* mutants (50,51). DLX5 can activate the *GnRH* promoter *in vitro* (Fig. 4) and is expressed in the appropriate spatiotemporal pattern relevant for the developing GnRH neuron (Fig. 1). Another potential explanation for the decreased numbers of GnRH-positive cells in the CNS of the *Dlx1&2*-null is improper migration. In fact, we observed a small portion of mis-migrating GnRH-positive cells as early as 13.5 dpc in these null embryos. Upon mis-migration, it is possible that these few neurons down-regulate expression of *GnRH* or apoptose in the absence of appropriate environmental cues.

The number of *GnRH*-positive cells in *Msx1*-null embryos was significantly greater than the number present in wild-type littermates on 13.5 dpc (Fig. 7, *panel D* and Table I). Intriguingly, the abnormality in the number and location of the GnRH population observed in the *Msx1*-null embryo is similar to that of mouse embryos with null mutations in *AP-2α*, which plays an important role in development of craniofacial features including the nasal environment of the GnRH neuron (52,53). Like MSX1, AP-2α co-localizes with GnRH-positive neurons as early as e13.5, but not in the adult (54) and *AP-2α*-null embryos show a considerable increase in the nasal population of GnRH-positive cells between 13.5 and 15.5 dpc, developmental stages when AP-2α co-expresses with GnRH in the wild-type embryo (55). In that study, expansion of the GnRH-positive nasal population was attributed to proliferation and misregulation of mitotic signals in the absence of AP-2α (55). Thus, an alternative hypothesis is that an increase in the population of GnRH-positive cells in the *Msx1* mutant represents a proliferation of the GnRH neuronal population, as MSX has also been implicated in cell cycle control (56). However, *Msx1* wild-type and null embryos showed no difference in the number of GnRH-positive cells also positive for the nuclear proliferation antigen, Ki67 (data not shown). Thus, the specific increase in *GnRH*-positive cells in the *Msx1*-null, rather than an actual increase in overall cell number, might also represent cells that express the *GnRH* transcript at levels below detection in a wild-type embryo and would be consistent with the role of *Msx1* as a transcriptional repressor of *GnRH* promoter activity.

Herein, we have found that the MSX and DLX transcription factor families are important for proper regulation of the *GnRH* promoter during development. Understanding the molecular identity of the GnRH neuronal population throughout development will facilitate a better understanding of the key factors involved in cell-specific as well as temporal expression of the *GnRH* gene. Such knowledge will also provide insight into the development of the hypothalamic-pituitary-gonadal axis and reproductive function.

Acknowledgments

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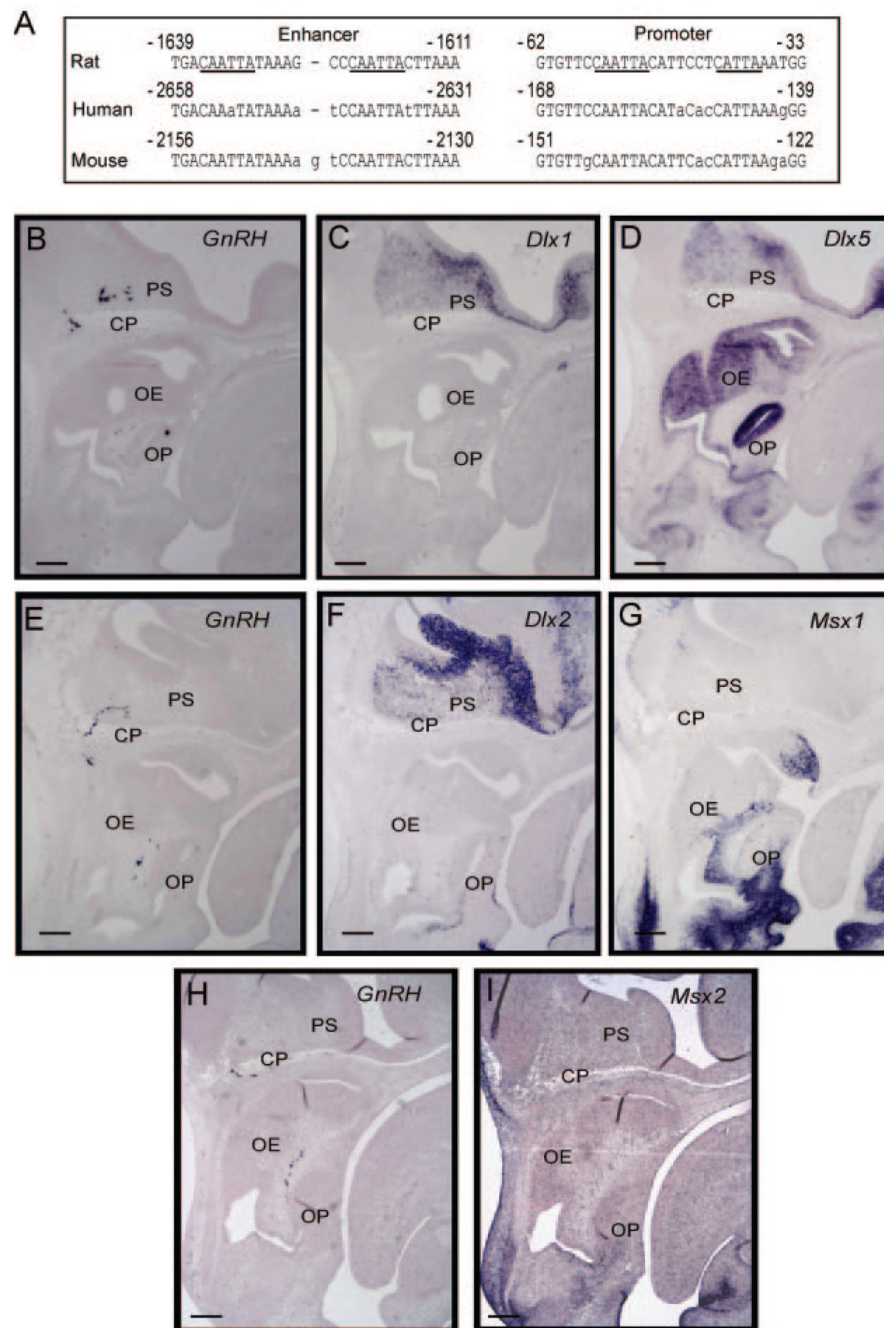56. Hu G, Lee H, Price SM, Shen MM, Abate-Shen C. Development 2001;128:2373–2384. [PubMed: 11493556]

Fig. 1. Conserved homeodomain binding sites in the *GnRH* upstream sequence and expression of candidate homeodomain regulators

Panel A, four consensus Q50 homeodomain (CAATTA) sites in the rat *GnRH* gene are located at −1636/−1631, −1623/−1618, −55/−50, and an additional CATTA site in the *GnRH* promoter at −42/−38 (*underlined* in the rat sequence). The homologous human and mouse *GnRH* sequences are shown with *lowercase letters* indicating mismatches from the rat sequence. *B*, expression of *Msx* and *Dlx* in relation to the migratory GnRH neuron *in vivo*. *In situ* hybridization was carried out on parasagittal sections from mouse embryos at 13.5 dpc and probed in series. Sense probes served as controls. Three separate embryos were used for the sections in series, grouped horizontally. Adjacent sections were hybridized using probes for

GnRH (*panel B*), *Dlx1* (*panel C*), and *Dlx5* (*panel D*), or *GnRH* (*panel E*), *Dlx2* (*panel F*), and *Msx1* (*panel G*), or *GnRH* (*panel H*), and *Msx2* (*panel I*). Abbreviations: olfactory epithelium (*OE*), cribriform plate (*CP*), olfactory bulb (*OB*), primordium of the septum (*PS*), olfactory placode (*OP*). *Black scale bars* represent 100 μm in the *lower left* of each panel.
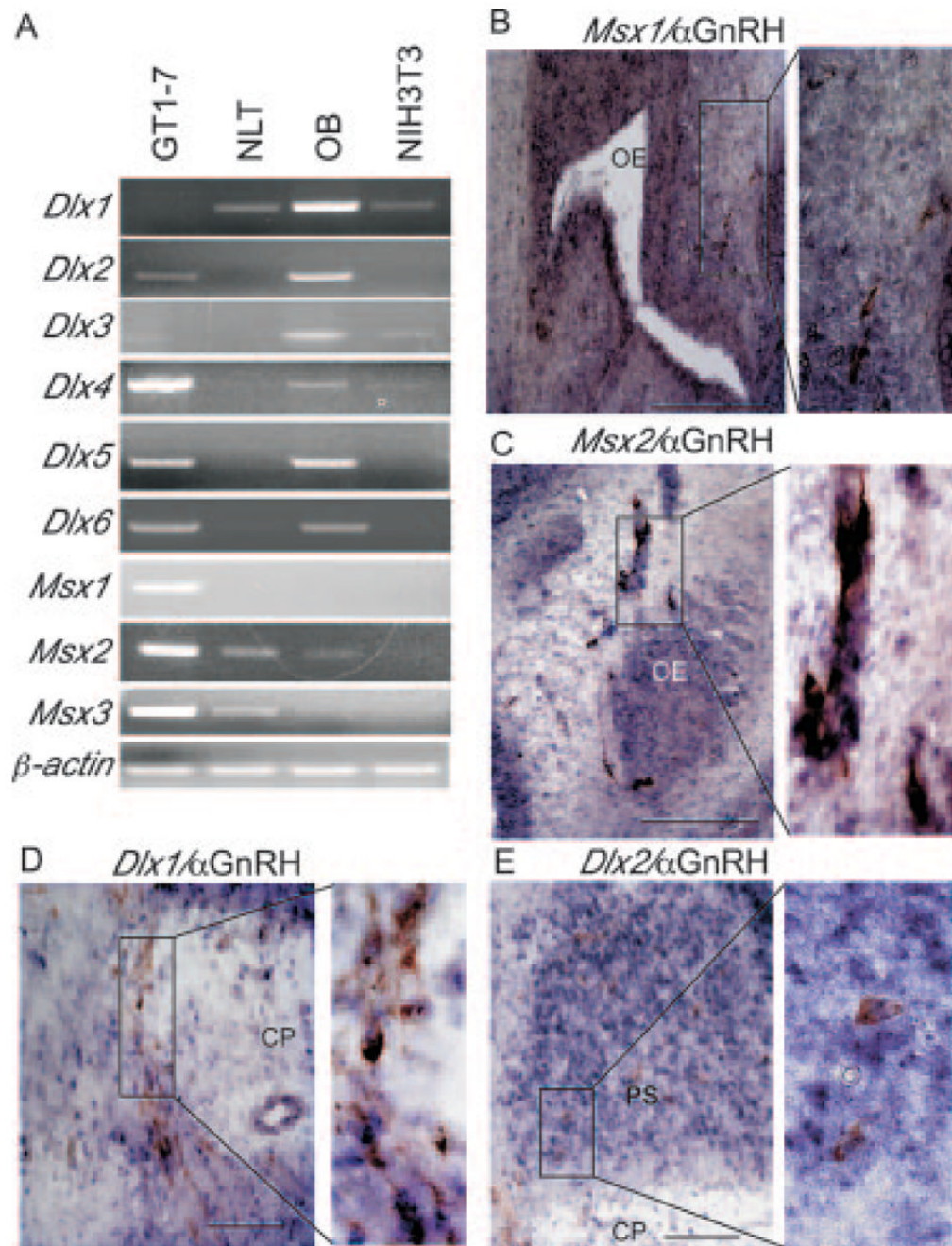
Fig. 2. Expression of Msx and Dlx family members in model GnRH cell lines and co-expression with GnRH *in vivo*

A, reverse transcriptase PCR analysis was used to identify the *Msx* and *Dlx* genes expressed in the GT1-7 and NLT cells. Olfactory bulb (*OB*) RNA was used as a positive control for the Dlx family members. NIH3T3 RNA is a mouse fibroblast control. *β*-Actin was used as a control for equal RNA loading. Ethidium-stained, 2% agarose gels are shown here. *B–E*, Msx and Dlx family members co-express with GnRH. Double *in situ* hybridization/immunohistochemistry was performed on parasagittal sections of 13.5-dpc embryos with an antibody recognizing GnRH (*brown*) and RNA probes (*blue*) for *Msx1* (*B*), *Msx2* (*C*), *Dlx1* (*D*), and *Dlx2* (*E*). The expanded panels to the *right* indicate higher magnification images of the boxed region to the

left. Abbreviations: olfactory epithelium (*OE*), cribriform plate (*CP*), primordium of the septum (*PS*), olfactory placode (*OP*). *Black scale bars* represent 100 μm in the *lower right* of each panel.
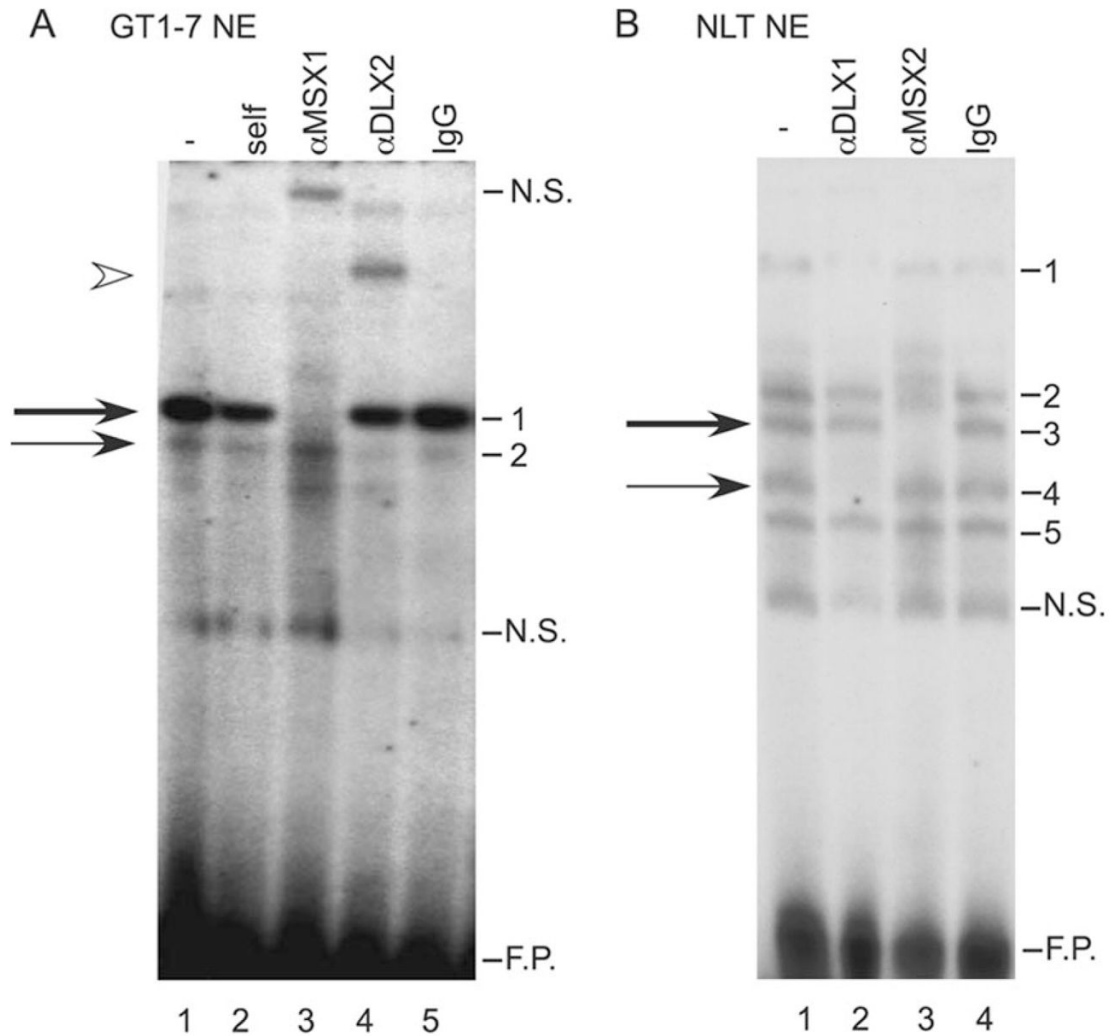
Fig. 3. MSX and DLX family members bind to the homeodo-main sites
A, EMSA analysis and antibody supershifts were performed using GT1-7 nuclear extract as well as antibodies recognizing MSX1, and DLX2 (*lanes 3* and *4*) and an IgG control (*lane 5*). The oligonucleotide probe corresponds to −1642/−1623 of the rat *GnRH* gene and contains the 5′ most homeodomain-binding site in the enhancer. The *thick arrow* marks the major complex and the *thin arrow marks* the minor complex. An *arrowhead* indicates the supershift of the minor complex. *B*, EMSA analysis and antibody supershifts were performed using the same oligonucleotide probe, NLT nuclear extract and an antibody recognizing DLX1 or MSX2 (*lanes 2* and *3*) or an IgG control (*lane 4*). The *thick arrow* represents the major complex and the *thin arrow* represents the minor complex. *N.S.* represents nonspecific binding, and *F.P.* indicates excess, free probe.
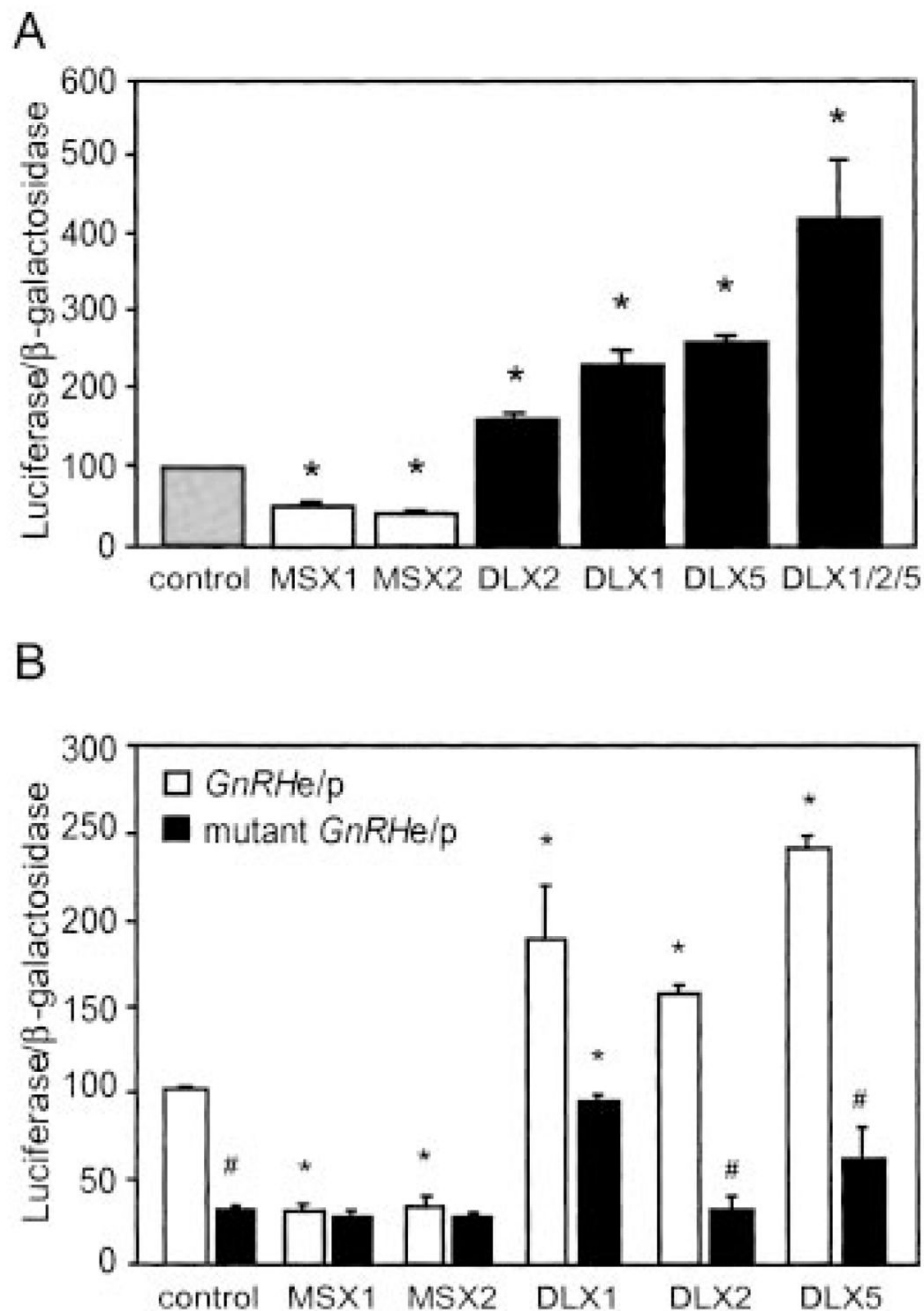
Fig. 4. Transcriptional activity of MSX and DLX on the *GnRH* gene in GT1-7 cells
A, the *GnRH*e/p-luciferase reporter was co-transfected with expression vectors for MSX1, MSX2, DLX1, DLX2, or DLX5 in transient transfection of GT1-7 cells. *B*, wild-type and mutant *Gn-RH*e/p luciferase reporters were co-transfected into GT1-7 cells with expression vectors for these same MSX and DLX family members. In both panels, a TK-*β*-galactosidase reporter was used to control for transfection efficiency. Percent activity is relative to empty vector control. *Asterisks* indicate statistical difference from the control and the pound sign (#) signifies difference between activity on the wild-type and mutant reporter by analysis of variance with Tukey-Kramer HSD ($p < 0.05$).
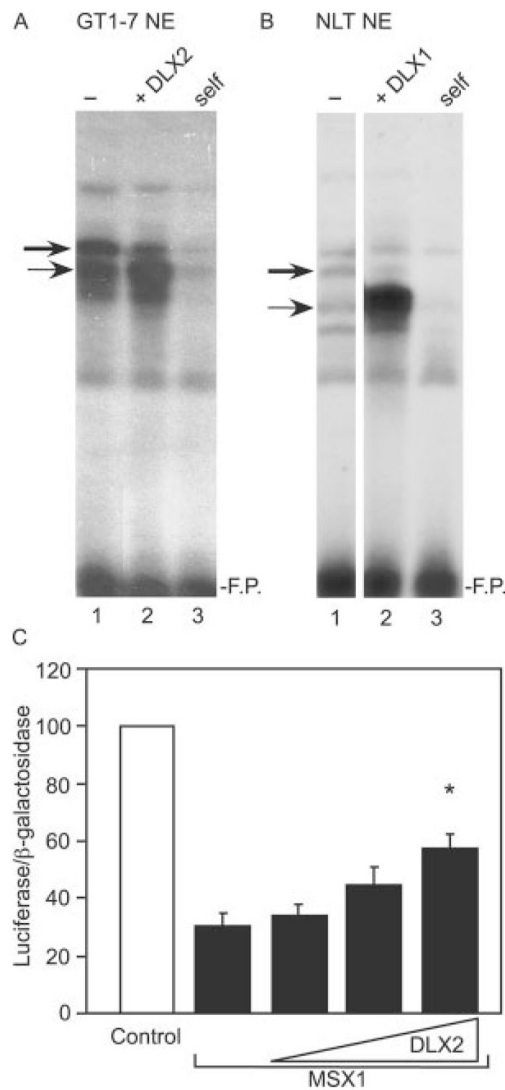
Fig. 5. Functional antagonism of MSX1 and DLX2 on the *GnRH* gene in GT1-7 cells
Competitive binding between MSX and DLX on the rat *GnRH* gene is shown. EMSA was performed with *A*, GT1-7 nuclear extract or *B,* NLT nuclear extract. The *thick arrow* represents the major complex (MSX) and the *thin arrow* represents the minor complex (DLX). *Lane 1* represents control nuclear extract, and *lane 2* shows extracts overexpressing DLX protein. *Lane 3* represents self-competition with 100× unlabeled oligonucleotide. The probe corresponds to the 5′-enhancer homeodomain binding site, −1642/−1623. *F.P.* represents excess, free probe. *C*, transient co-transfection of the GT1-7 cells was performed with the *GnRH*e/p-luciferase reporter and a plasmid vector overexpressing *MSX1* in addition to 1×, 2×, and 3× the amount of an expression vector for DLX2. Percent activity is relative to empty vector control. The *asterisk* indicates statistical difference from MSX1 alone by analysis of variance with Tukey-Kramer HSD ($p < 0.05$).
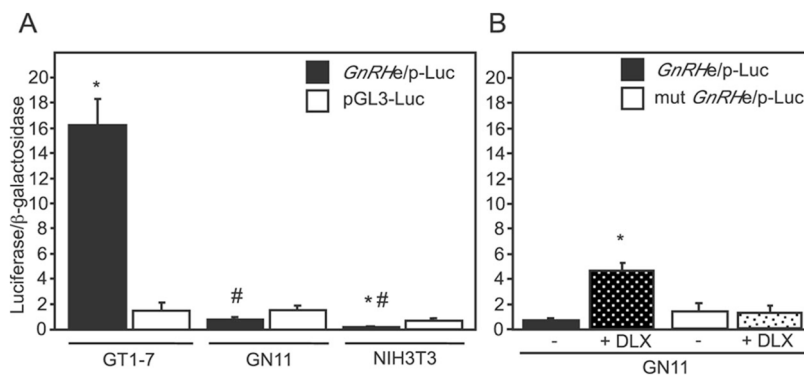
Fig. 6. Relative *GnRH* expression in model cell lines
A, transient transfection of the *GnRH*e/p-luciferase reporter was performed in GT1-7, GN11, and NIH3T3 cells. In addition, an RSVe/RSVp-*β*-galactosidase reporter was included to normalize for transfection efficiency. All values are normalized to the RSVe/RSVp-luciferase to control for metabolic differences in the cell lines. The *asterisk* indicates statistical difference from the pGL3-luc control while the pound sign indicates statistically significant difference from the *GnRH*e/p across cell lines by analysis of variance with Tukey-Kramer HSD ($p < 0.05$). *B*, overexpression of DLX family members in GN11 cells enhances promoter activity. Transient transfection of GN11 cells was performed with the *GnRH*e/p or mutant *GnRH*e/p in concert with expression vectors for DLX1, -2, and -5 (represented as + DLX). The *asterisk* indicates statistical difference from the empty vector control.
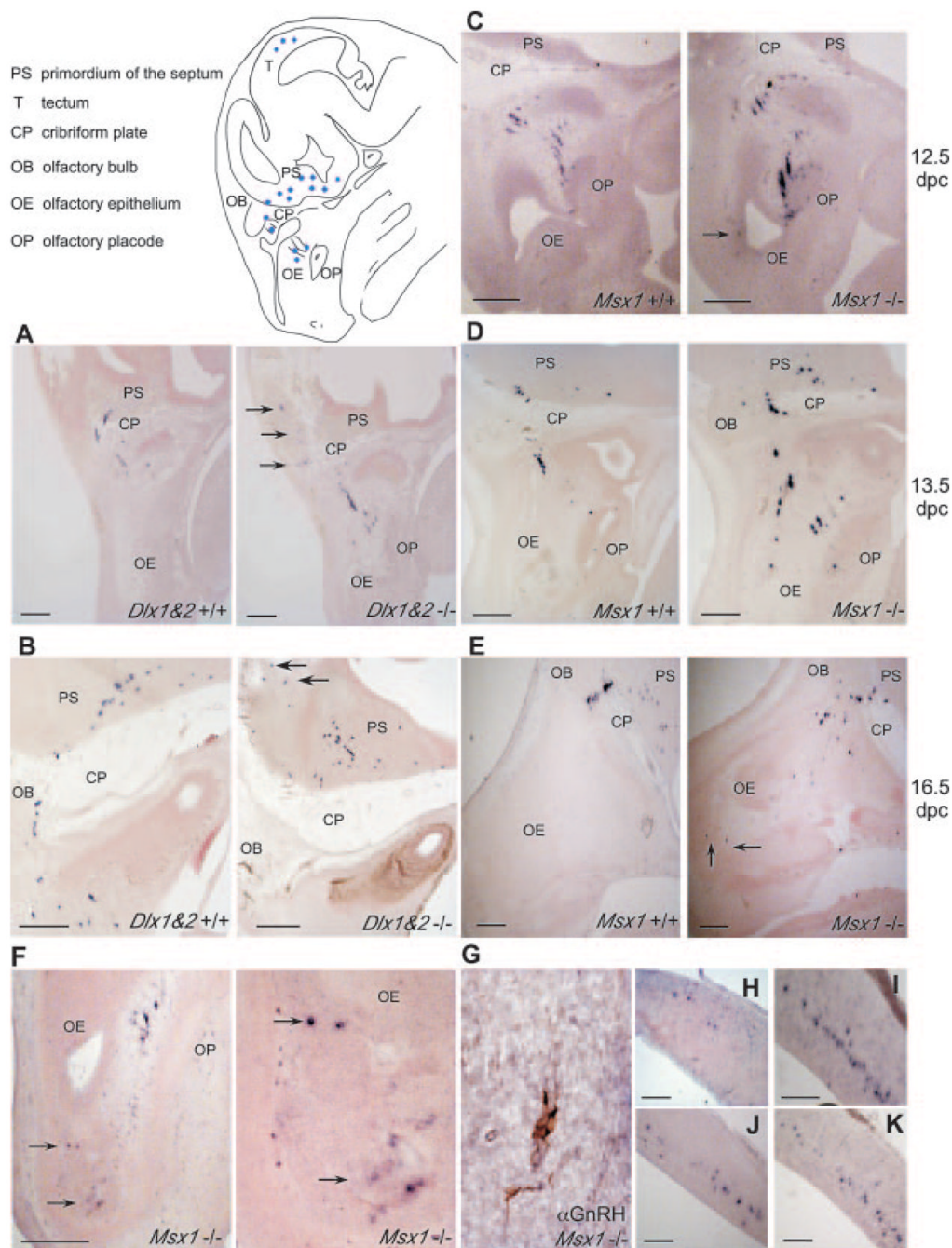
Fig. 7. GnRH-positive cells in the *Dlx1&2* or *Msx1*-null mouse embryos

In situ hybridization of *Dlx1&2* or *Msx1*-positive and null embryonic mice ages 12.5, 13.5, and 16.5 dpc was carried out as described using a DIG-*GnRH* probe. Images represent parasagittal sections of the wild-type (*left panel*) and null (*right panel*) embryos from 13.5-dpc *Dlx1&2* (*panel A*), 16.5-dpc *Dlx1&2* (*panel B*), 12.5-dpc *Msx1* (*panel C*), 13.5-dpc *Msx1* (*panel D*), and 16.5-dpc *Msx1* (*panel E*). *Panel F*, 13.5-dpc *Msx1*-null embryo with GnRH-positive cells in the olfactory epithelium (*arrow*) at low (*left panel*) and higher (*right panel*) magnification are indicated. *Panel G*, immunohistochemical analysis of GnRH-positive cells located in the olfactory epithelium of *Msx1*-null embryos at 13.5 dpc. GnRH-positive cells located in the tectum of 13.5-dpc *Msx1*-null (*panel H*), 16.5-dpc *Dlx1&2*-null (*panel I*), 16.5-

dpc wild-type (*panel J*), and 16.5-dpc *Msx1*-null (*panel K*) embryo. *Arrows* indicate GnRH-positive cells located outside of the characterized migratory route for the septohypothalamic population. *Black scale bars* represent 100 *µ*m in the *lower left* of each panel. The schematic represents a parasagittal view of a mouse embryo head in the region where GnRH-positive neurons are found.

Table I

GnRH-positive cells counted by in situ hybridization of mouse embryos

In situ hybridization was performed using an antisense RNA probe for *GnRH* to assess the number of *GnRH*-positive cells. Whole heads of mouse embryo littermates were sectioned, hybridized, and then counted for *GnRH*-positive cells.

Genotype		Embryo age	Nose	Primordium of the septum	Tectum	Total (septohypothalamic)
		dpc				
Dlx1&2	+/+	13.5	668 ± 74	277 ± 92	0	945 ± 62
Dlx1&2	−/−	13.5	515 ± 125	97 ± 60	0	612 ± 177
Dlx1&2	+/+	16.5	153 ± 40	833 ± 34	203 ± 29	985 ± 16
Dlx1&2	−/−	16.5	72 ± 36	610 ± 45	204 ± 18	681 ± 81
Msx1	+/+	12.5	733 ± 15	125 ± 3	0	858 ± 18
Msx1	−/−	12.5	896 ± 52	137 ± 14	0	1,032 ± 54
Msx1	+/+	13.5	710 ± 44	229 ± 31	0	939 ± 20
Msx1	−/−	13.5	1,021 ± 99	267 ± 25	19 ± 6	1,300 ± 116
Msx1	+/+	16.5	309 ± 58	728 ± 63	238 ± 26	1,037 ± 103
Msx1	−/−	16.5	227 ± 49	643 ± 64	215 ± 31	871 ± 53