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Opioid Receptor-Induced GTPγ^{35}S Binding during Mouse Development

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Although a large superfamily of G-protein-coupled receptors serves multiple functions, little is known about their functional activation during ontogeny. To examine the functional activation of the mu-opioid receptor (MOR) and the delta-opioid receptor (DOR) during development, sections of mouse embryos and fetuses from e11.5 until birth were treated with DAMGO and DPDPE, respectively, and the ability of these drugs to induce G-protein coupling was assessed by using GTPγ^{35}S binding autoradiography. MOR activation was first detected in the caudate-putamen (CPU) at e12.5, and by e15.5, activity had not only increased in this region but also expanded to include the midbrain, medial habenula, hypothalamus, pons, and medulla. DOR activity first appeared at e17.5 in the hypothalamus, pons, medial habenula, and medulla and at p1 in the CPU at levels noticeably less than those of the MOR. In general, MOR and DOR activation lagged only slightly behind the appearance of MOR-1 and DOR-1 mRNA but delayed activation was particularly pronounced in the trigeminal ganglia, where MOR-1 gene expression was first detected at e13.5, but MOR activity was not observed even at birth. Thus, the data demonstrate temporal and often region-specific differences in the appearance and magnitude of functional activity in cell groups expressing either the MOR-1 or DOR-1 genes, suggesting that interaction between the opioid receptors, G-proteins, and other signaling cofactors is developmentally regulated.

Key Words: **embryonic; development; opiate; opioid receptors; GTPγ^{35}S binding autoradiography; G-protein coupling.**

INTRODUCTION

G-protein-coupled receptors (GPCR) comprise a large superfamily of receptors with over 1000 members discovered to date. These receptors participate in the actions of a variety of ligands, including biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions, and proteases, and participate in the perception of external stimuli, such as light, odor, and taste (Kolakowski, 1994; Mombaerts, 1999). Current knowledge indicates that GPCR are involved in a myriad of biological processes both in the CNS and throughout the organism.

Embryonic development of the nervous system is one process in which the involvement of GPCR warrants investigation. One group of GPCR that has received significant attention in this context is the opioid receptor family. A role for the opioid system in the development of both nervous and somatic structures has been suggested based on studies showing many detrimental effects of perinatal exposure to both morphine and methadone, including lowered survival rates (Eriksson and Ronnback, 1989; Hutchings *et al.*, 1992), decreased body weight (Ford and Rhines, 1979; Eriksson and Ronnback, 1989; Kunko *et al.*, 1996), impeded brain growth (Zagon and McLaughlin, 1977; Ford and Rhines, 1979), delayed appearance of neuromuscular reflexes and behaviors (Zagon and McLaughlin, 1978; Zagon *et al.*, 1979a,b; Kunko *et al.*, 1996), restricted neuronal proliferation (Seatriz and Hammer, 1993), and physical dependence (Zagon and McLaughlin, 1981; Seatriz and Hammer, 1993). Chronic administration of the general opiate antagonist naltrexone to newborn pups or pregnant mice was initially shown to produce several interesting phenotypes, including increased weight, enlarged brains, increased cerebellar volume, an acceleration in the appearance of sensorimotor behaviors, and increased Purkinje cell dendritic arborization (Zagon and McLaughlin, 1983, 1985, 1987; Zagon, 1987). More recent studies have shown that continuous opioid receptor blockade for the duration of gestation results in even greater increases in body, brain, and organ growth (McLaughlin *et al.*, 1997b), accelerates the appearance of physical characteristics, reflexes, and spontaneous motor behaviors (McLaughlin *et al.*, 1997a), and produces a decrease in MOR binding density and decreased sensitivity to the analgesic effects of morphine (Zagon *et al.*, 1998).

The effect of the opioid system on development is also of clinical interest as many human infants born to either actively abusing or methadone-maintained heroin addicted mothers are exposed to opiates *in utero*. A prospective study of human opiate-exposed infants revealed that at 1

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year postnatally these children are at an increased risk for mild impairment in psychomotor development (Bunikowski *et al.,* 1998). Understanding how the opioid receptors function during the fetal period will thus be useful in assessing the mechanism by which *in utero* opiates produce these and other negative outcomes.

The region-specific appearance of messenger RNA for the three classical opioid receptors (MOR-1, DOR-1, and KOR-1) has been described in both the nervous system and peripheral tissues of the embryonic and fetal mouse (Zhu *et al.,* 1998), consistent with the possibility that these receptors influence fetal development. Although the prenatal appearance of opioid receptor binding in whole brain homogenates has been detected (Rius *et al.,* 1991), an anatomical comparison of opioid receptor proteins with the RNA distribution during prenatal development is still lacking. Although tissue section autoradiography with the nonspecific ligand [^{3}H]naloxone first detected opioid receptors in the rat at e14 in the striatum and at e16 in the olfactory cortical primordium and medial septum (Kent *et al.,* 1981), the prenatal distribution of specific opioid receptor subtypes has not been explored at the protein level.

The construction of several genetic models of the opioid system, including individual and combinatorial mutations of each of the classical opioid receptors using gene targeting, has shown that mouse strains bearing one, two, or even three null alleles of the opioid receptors show no obvious developmental abnormalities (Simonin *et al.,* 1998; Schuller *et al.,* 1999; Zhu *et al.,* 1999; Gaveriaux-Ruff *et al.,* 2001; Clarke *et al.,* 2002). While thorough cell counts and structural analysis of opioid receptor mutant mice are still in progress, the grossly normal phenotype of the mutant mice raises the question of whether the opioid receptor proteins can be activated during development, which is critical to determining whether the detrimental effects of opiates and naltrexone on the developing nervous system could result from nonspecific effects.

Conclusions drawn from gene expression or ligand binding studies examining opioid system development are limited to the extent that neither provide functional assessment of receptor activity. With the advent of GTPγ^{35}S binding assays (Hilf *et al.,* 1989; Lorenzen *et al.,* 1993; Traynor and Nahorski, 1995) and its adaptation to autoradiography (Sim *et al.,* 1995), it is now possible to determine the anatomical pattern of opioid receptor coupling to G-proteins in histological sections. The experiments described below use these techniques to extend previous gene expression and ligand binding studies and ascertain at what stage and in which fetal brain regions the mu opioid receptor (MOR) and delta opioid receptor (DOR) become active and how the onset of this functional parameter of receptor activity is related to gene activation. Such data can determine whether the opioid receptors are active during development and provide a clearer picture of when and where the opioid receptors are able to influence the nervous system and possibly other tissue types.

METHODS

Tissue Preparation

All studies were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for Care and Use of Experimental Animals. Fresh frozen sections were used in all experiments. All mice were quickly staged according to their limb bud morphology before being embedded and frozen in OCT for cryostat sectioning. The following age groups were examined: e11.5, e12.5, e13.5, e15.5, e17.5, p1. Two mice were used from stage e11.5 and e12.5, while three mice were used from stage e13.5 onward. Transverse sections through the head were prepared from e11.5 embryos. Coronal sections were prepared from the heads of embryos from all other stages. In all cases, 20-μm thick sections were cut, mounted to TESPA subbed slides, and stored at –70°C until use.

GTPγ^{35}S Autoradiography

Previous experiments (Sim and Childers, 1997) have confirmed the specificity of DAMGO for the MOR and DPDPE for the DOR in this paradigm. In addition, experiments performed in MOR-1 and DOR-1 KO adult membrane homogenates revealed a total lack of DAMGO- and DPDPE-induced GTPγ^{35}S binding, respectively, at the concentrations used here for autoradiography, further supporting the specificity of these drugs (data not shown). Thus, DAMGO-induced GTPγ^{35}S binding was used to assess MOR coupling, and DPDPE-induced GTPγ^{35}S binding was used to assess DOR coupling. DAMGO- and DPDPE-induced GTPγ^{35}S binding dose response curves were constructed in adult wild type mouse whole brain homogenates. Concentrations of agonist producing maximal effects in these membrane assays were used in all autoradiography experiments.

GTPγ^{35}S binding autoradiography was carried out according to protocols developed elsewhere (Sim *et al.,* 1995; Sim and Childers, 1997) with some modifications. Gly–gly was substituted for Tris–HCl as a buffering agent, and DTT was added to the assay buffer. These modifications have been shown to increase the signal-to-noise ratio of the assay (data not shown). Triplicate sections were first equilibrated in assay buffer (50 mM Gly–Gly, 2 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.2 mM DTT) for 10 min. Sections where then incubated in assay buffer with 2 mM GDP for 15 min. Finally, sections were incubated in assay buffer with 2 mM GDP, 0.04 nM GTPγ^{35}S, and 3 μM DAMGO (to assess MOR G-protein coupling), or 10 μM DPDPE (to assess DOR G-protein coupling) for 2 h. Basal GTP binding was determined by incubating the sections in the absence of the agonist.

Incubations in histological sections were ended by rinsing twice with ice-cold 50 mM Gly–Gly and 0.2 mM DTT for 2 min each and rinsing once in ice-cold distilled water for 30 s. After drying, the slides containing the brain sections were exposed to BioMax MR X-ray film for 5–30 days. Basal binding from all embryonic sections was considerably less than that observed in adult mice, indicating little spontaneous G-protein activity. This allowed for long exposure times and the ability to detect the small yet significant levels of agonist-induced GTPγ^{35}S binding at earlier embryonic ages. For reasons that remain unknown, cartilaginous structures of the head and trunk often show high levels of basal GTPγ^{35}S binding (labeled "c" in Figs. 1A, 1B, 2A, 2B, 5E, and 5F) which are not significantly increased by agonist treatment, indicating nonspecific binding of the GTPγ^{35}S to these areas.

The images of the individual brain section were then digitized by using a Polaroid Digital Microscope Camera model Ie and accompanying software. Each embryo of each stage was analyzed densi-

tometrically by using the NIH Image software package. The density of GTPγ^{35}S binding was converted to nCi/g using [^{14}C] standards enclosed in the film cassette. For each animal, in each brain region, nCi/g values from three agonist-stimulated sections were compared with three basal sections by using a *t* test to determine whether significant coupling was evident. Coupling was not considered present in a brain region unless significant coupling was seen in all embryos assessed at each stage.

RESULTS

GTPγ^{35}S binding autoradiography was used to determine at what stage and in which brain regions the MOR and DOR are functionally active as assessed by the ability to couple to G-proteins. As described below, there were region-specific differences in the appearance of functional activity by cell groups expressing either the MOR-1 or DOR-1 genes and, in many cases, the appearance of detectable receptor activity lagged one or more days behind the onset of gene expression. Summaries of the temporal and spatial data for both MOR activation and DOR activation are presented in Tables 1 and 2, respectively, which include both semiquantitative and quantitative measurements. For clarity, the results are presented by age.

e11.5

Although MOR-1 mRNA is first detected in the differentiating cells of the basal ganglia at this stage (Zhu *et al.*, 1998), no detectable MOR receptor coupling was detected, even after 1 month of film exposure (see Methods). No DOR receptor coupling was seen at this stage, which is consistent with the previously reported lack of detectable gene expression at this stage (Zhu *et al.*, 1998).

e12.5

At e12.5, DAMGO-induced GTPγ^{35}S binding was first observed in the caudate-putamen (CPU) (Figs. 1A and 1B), which coincides with the pattern of MOR-1 gene expression at this stage. This result indicates that all factors needed for Gi/Go coupling are present in these cells by this age. Interestingly, while no other brain region showed receptor coupling, MOR-1 mRNA is detected in other regions, including the midbrain and hindbrain at this age (Zhu *et al.*, 1998), indicating that there is a mismatch between the time of initial MOR-1 gene expression and detectable receptor activation in these regions. No DPDPE-induced GTPγ^{35}S binding was seen at this age, again consistent with the lack of detectable DOR-1 gene expression at this stage (Zhu *et al.*, 1998).

e13.5

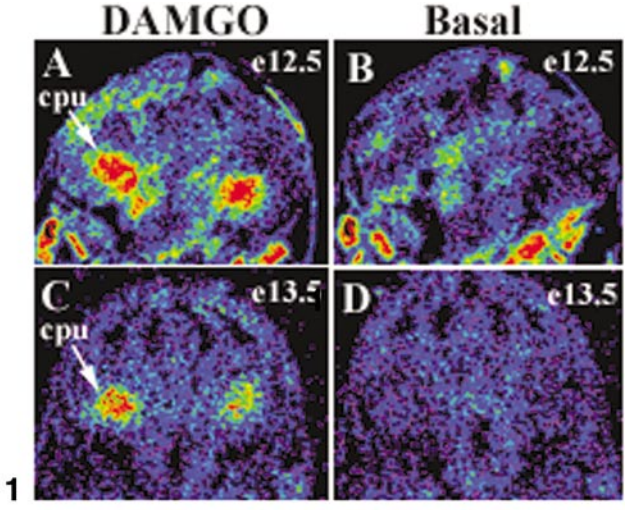
At e13.5, the opioid receptor activation pattern is similar to that at e12.5. DAMGO-induced GTPγ^{35}S binding is again found only in the CPU (Figs. 1C and 1D), while MOR-1 gene expression has expanded to include midbrain, medial habenula, hypothalamus, pons, medulla, and the trigeminal ganglia. However, expression levels in these regions are noticeably less than those seen in the CPU (Zhu *et al.*, 1998). DOR-1 gene expression first appears in this stage and is confined to the hypothalamus and pons (Zhu *et al.*, 1998), although no detectable DPDPE-induced GTPγ^{35}S binding is present. Thus, at this stage, there are mismatches between gene expression and detectable receptor activation for both the MOR and DOR in several brain regions. One mismatched region of particular interest is the trigeminal ganglion, which shows very high levels of MOR-1 gene expression, similar to the levels in the CPU (Zhu *et al.*, 1998), but no detectable GTPγ^{35}S binding at this stage. As there is detectable DAMGO-induced coupling in the CPU at e13.5, the MOR is clearly able to influence the function of these cells at this early stage. However, the scope of its influence appears to be less than the gene expression data would suggest.

e15.5

At e15.5, detectable DAMGO-induced GTPγ^{35}S binding expands significantly to several other brain regions, including the midbrain (Figs. 2G and 2H), medial habenula (Figs. 2C and 2D), hypothalamus (Figs. 2C and 2D), pons (Figs. 2E and 2F), and medulla (Figs. 2G and 2H). In addition, MOR-1 gene expression levels increase in nearly all regions that previously showed expression, although none achieve the high levels seen in the CPU (Zhu *et al.*, 1998). Thus, at this stage, the expansion of MOR coupling parallels the changes in gene expression and eliminates the mismatch between gene expression and receptor activity in many brain regions. However, a mismatch remains in the trigeminal ganglion, which still shows no detectable DAMGO-induced GTPγ^{35}S binding, despite high levels of mRNA (Figs. 2C and 2D). A similar mismatch also remains for the DOR, as DPDPE-induced GTPγ^{35}S binding is still undetectable at this stage, while gene expression is detectable in the pons and hypothalamus.

e17.5

The pattern of DAMGO-induced GTPγ^{35}S binding at e17.5 remains relatively unchanged compared with e15.5. However, DAMGO-induced GTPγ^{35}S binding first appears in the olfactory bulb (OB) (Figs. 3A and 3B). As MOR-1 gene expression also begins in the OB at this stage (Zhu *et al.*, 1998), the onset of MOR coupling exactly coincides with the appearance of its mRNA in this region. In addition, MOR coupling in the trigeminal ganglion remains undetectable at this stage (Figs. 3G and 3H). DPDPE-induced GTPγ^{35}S binding is detected for the first time and is restricted to the hypothalamus (Figs. 3H and 3I), pons (Figs. 3K and 3L), medulla (Figs. 3N and 3O), and medial habenula (Figs. 3G and 3H). As DOR-1 gene expression is first seen in the pons and hypothalamus at e13.5 (Zhu *et al.*, 1998), there is a 4-day lag between the appearance of DOR-1 mRNA and functional activation in these regions. Interestingly, the detection of DOR coupling in the medulla and medial habenula occurs in the absence of detectable gene expression of the cognate receptors at this stage (Zhu *et al.*, 1998), which may reflect high levels of translation and coupling from relatively low levels of DOR-1 mRNA.



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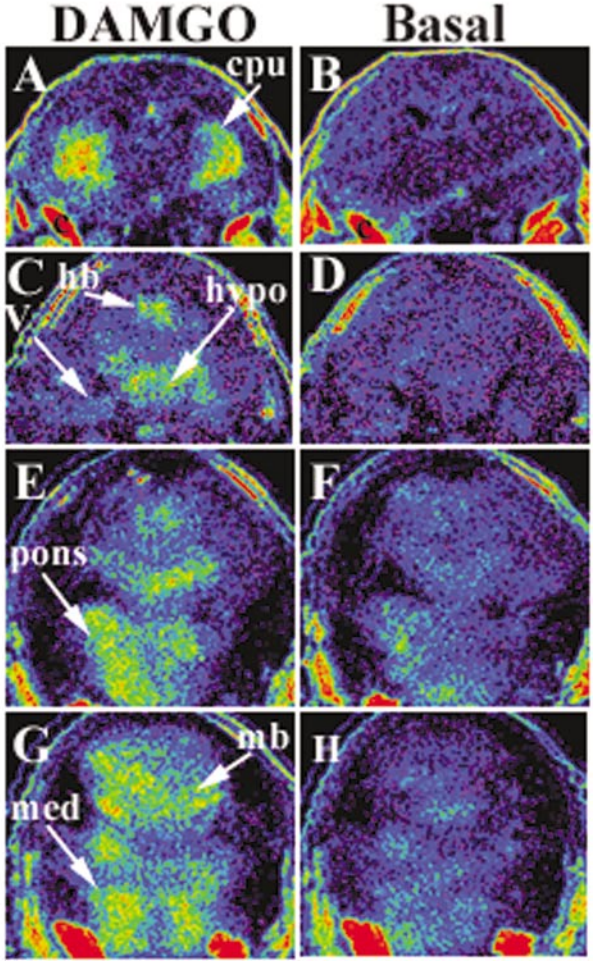

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FIG. 1. DAMGO-stimulated GTPγ^{35}S binding in coronal sections of e12.5 and e13.5 mouse embryos at the level of the caudate-putamen. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DAMGO for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. cpu, caudate-putamen; c, cartilage.

p1

The pattern of MOR receptor activation at p1 is very similar to that seen at e17.5. The main difference is that MOR coupling can now be localized to more discrete regions of the midbrain, such as the substantia nigra (SN) and the periaqueductal gray (PAG) (Figs. 4G and 4H). At this stage, except for the trigeminal ganglia (Figs. 4E and 4F), all regions that show MOR-1 gene expression also show MOR receptor coupling. In addition, DOR coupling also appears in the CPU (Figs. 5A and 5B). As DOR-1 gene expression was first seen in the CPU at e17.5, there is a 2-day lag between the beginning of expression and onset of receptor function in this region.

DISCUSSION

In this study, we provide the first broad analysis of GPCR function during embryonic development. By assessing the developmental regulation of MOR and DOR activation, the studies presented here extend both previous gene expression studies (Zhu *et al.*, 1998) and more limited studies of protein expression (Kent *et al.*, 1981; Rius *et al.*, 1991) by determining the stage and brain regions where the factors necessary for opioid receptor signal transduction appear during ontogeny. The data show that both the MOR and DOR are active during the prenatal and early postnatal period and are positioned to impact the development and function of the nervous system. Nonetheless, in nearly all areas studied there is some mismatch between receptor gene expression and receptor activity lasting one or more days, suggesting that production of G-proteins or other cofactors necessary for opioid receptor signal transduction may also be developmentally regulated.

Summary of Receptor Activation and Comparison with Adult Sites of Activation

The developmental pattern of MOR coupling is summarized in Table 1. Functional activity is first detected at e12.5 with coupling restricted to the CPU. Activation remains restricted to this region until e15.5, when coupling increases in the CPU and expands to include the midbrain, medial habenula, hypothalamus, pons, and medulla. At the end of the embryonic period, the pattern of MOR coupling closely resembles the adult pattern with some notable

FIG. 2. DAMGO-stimulated GTPγ^{35}S binding in coronal sections of e15.5 mouse embryos. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DAMGO for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. Sections were taken at the level of the caudate-putamen (A, B), hypothalamus (C, D), pons (E, F), and medulla (G, H). cpu, caudate-putamen. hb, medial habenula; hypo, hypothalamus; med, medulla; V, trigeminal ganglion; c, cartilage.

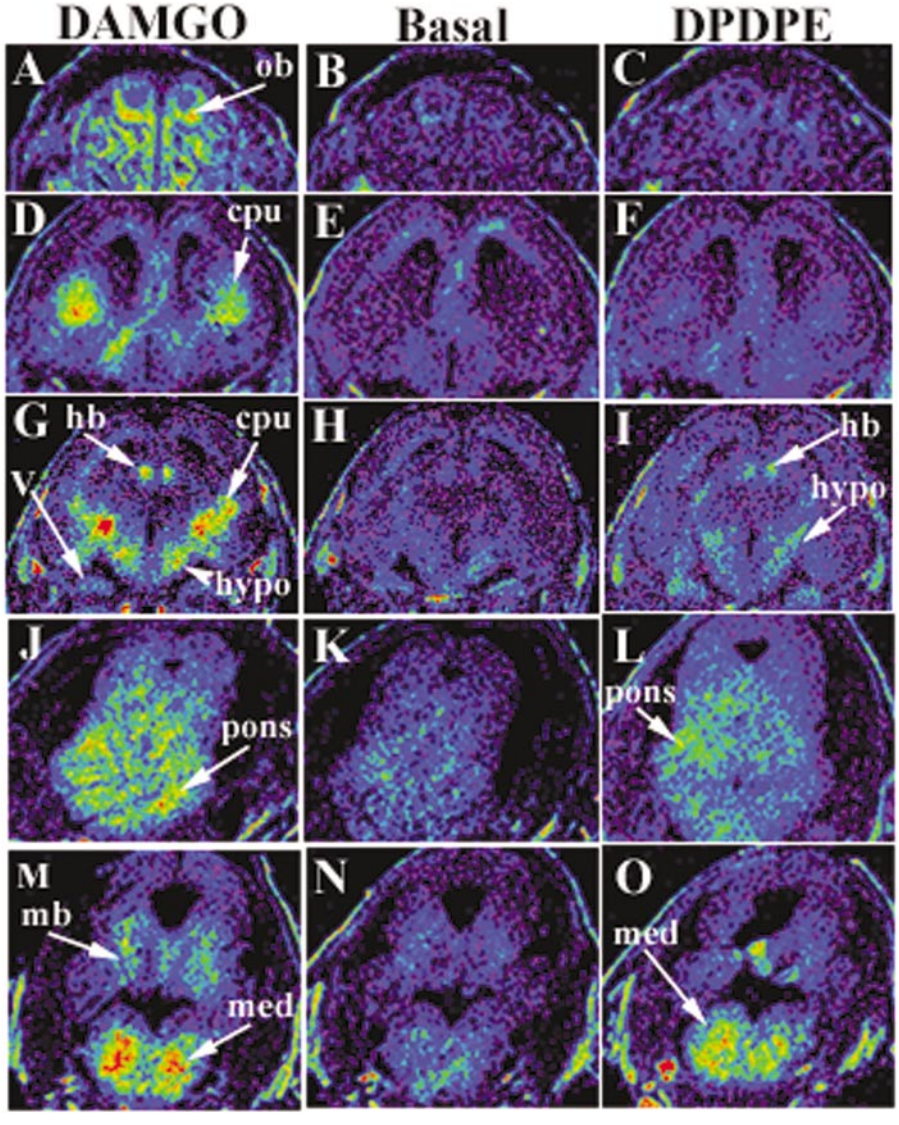

FIG. 3. DAMGO- and DPDPE-stimulated GTPγ^{35}S binding in coronal sections of e17.5 mouse embryos. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DAMGO for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. Sections were taken at the level of the olfactory bulb (A–C), caudate-putamen (D–F), hypothalamus (G–I), pons (J–L), and medulla (M–O). ob, olfactory bulb; cpu, caudate-putamen; hb, medial habenula; hypo, hypothalamus; med, medulla; V, trigeminal ganglion.

exceptions. First, except for the medial habenula, MOR coupling, as well as MOR-1 gene expression (Zhu *et al.*, 1998), is entirely absent from the thalamus during the prenatal period. In contrast, several thalamic nuclei show considerable MOR coupling and gene expression in the adult rodent (Mansour *et al.*, 1994; Sim and Childers, 1997), suggesting that although the MOR has a large role in the functioning of these structures in the adult brain, MOR appears to have a minimal, if any, role in their functioning during development.

The developmental pattern of DOR coupling is summarized in Table 2. In contrast to the widespread and high levels of MOR coupling seen throughout the embryonic period, DOR coupling appears relatively late and shows a much more restricted distribution. DOR coupling is first seen at e17.5 in the hypothalamus, pons, medial habenula, and medulla. By p1, DOR activation has expanded to include the CPU. In all of these regions, levels of coupling were noticeably lower than that of the MOR and generally reflected relative levels of the cognate mRNA. As with the MOR, the pattern of receptor coupling at the end of the prenatal period is similar to that seen in the adult, with some exceptions. DOR coupling and DOR-1 mRNA are absent at birth from the amygdala, which shows relatively

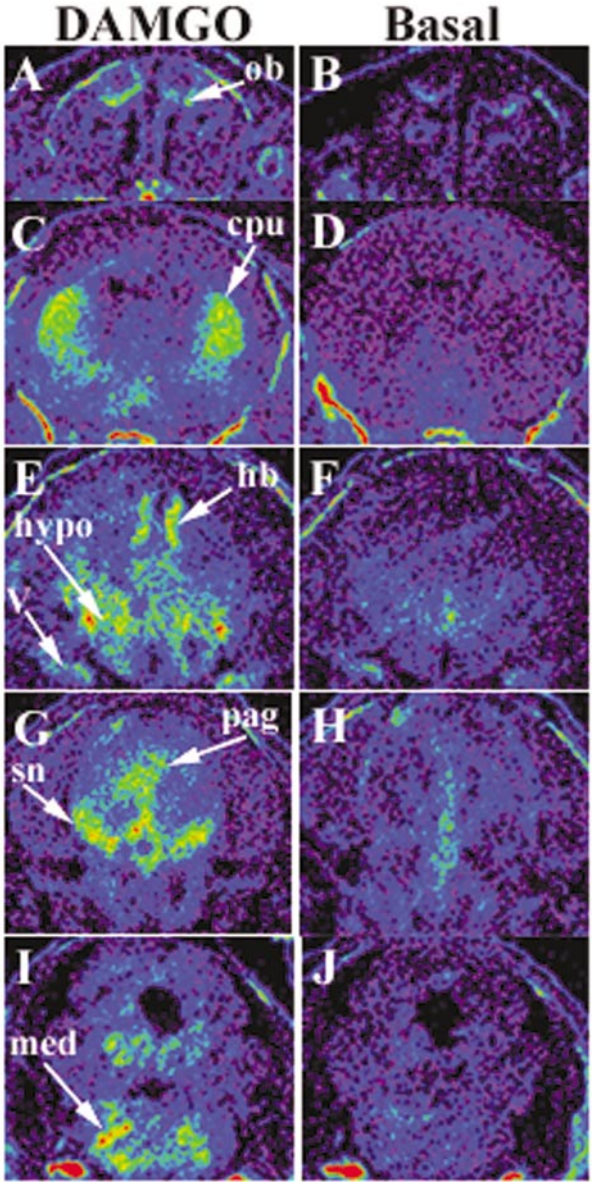

FIG. 4. DAMGO-stimulated GTPγ^{35}S binding in coronal sections of p1 mice. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DAMGO for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. Sections were taken at the level of the olfactory bulb (A, B), caudate-putamen (C, D), hypothalamus (E, F), substantia nigra (G, H), and medulla (I, J). ob, olfactory bulb; cpu, caudate-putamen; hb, medial habenula hypo, hypothalamus; sn, substantia nigra; pag, periaqueductal gray; med, medulla; V, trigeminal ganglion.

high levels of DOR coupling and gene expression in the adult rodent (Mansour *et al.*, 1994; Sim and Childers, 1997). Although the DOR appears to play a role in the functioning of this region in the adult (Filliol *et al.*, 2000), based on the studies presented here, it has little impact on the prenatal function of this structure.

Temporal Comparison between Opioid Receptor Gene Expression and Activation

MOR receptor coupling first appears at e12.5 in the CPU, which lags 1 day behind the appearance of its mRNA at e11.5 (Zhu *et al.*, 1998). Although only a short lag between the onset of gene expression and receptor coupling is seen in the CPU, 2- or 3-day lags are seen for the MOR in nearly all other brain regions assessed, including the midbrain, medial habenula, hypothalamus, and pons (Table 3). Although rather high levels of mRNA were detected in the trigeminal ganglion as early as e13.5 (Zhu *et al.*, 1998), we have found no evidence of MOR coupling during the entire fetal period, which may limit the ability to modulate pain. In general, the lag between the appearance of DOR-1 gene expression and receptor activation was longer than for MOR. Although the 1- or 2-day lag seen for the DOR in the CPU is similar to the lag seen in that brain region for MOR, the pons and hypothalamus showed longer delays (see Table 3). For the DOR, a 4-day lag was found in the pons and hypothalamus,

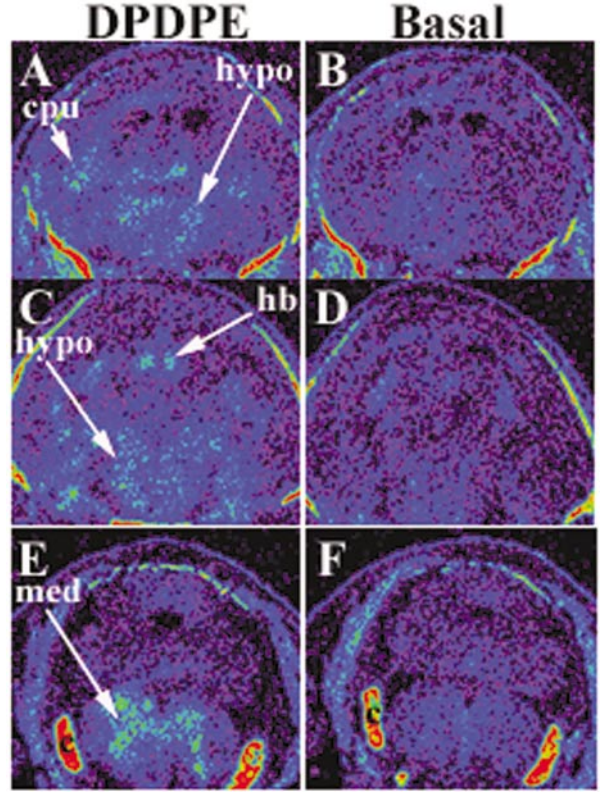

FIG. 5. DPDPE-stimulated GTPγ^{35}S binding in coronal sections of p1 mice. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DAMGO for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. Sections were taken at the level of the caudate-putamen (A, B), hypothalamus (C, D), and medulla (E, F). cpu, caudate-putamen; hb, medial habenula; hypo, hypothalamus; med, medulla; c, cartilage.

while for the MOR, a 3-day lag was seen in the pons and a 2-day lag in the hypothalamus, indicating slower maturation of DOR coupling during development.

The mismatch observed between gene expression and receptor function could be explained in several ways: (1) limited assay sensitivity, (2) the presence of low level of receptor protein preceding detectable gene expression, and (3) absence of appropriate G-proteins and other cofactors necessary for coupling. Although a lack of sensitivity may explain the mismatch in some regions, it is unlikely to be responsible for the mismatch in regions with high levels of mRNA expression, which would presumably also have high levels of receptor. In the CPU, the high levels of MOR-1 gene expression beginning at e11.5 (Zhu *et al.*, 1998) quickly lead to MOR coupling at e12.5; however, clearly this is not the case for the medulla and trigeminal ganglia. Gene expression in both of these regions begins at e13.5 (Zhu *et al.*, 1998) at levels similar to those seen in the CPU. However, MOR coupling is not detected until e15.5 in the medulla and is not detected at all during the embryonic period in the trigeminal ganglia. Thus, in these regions, a lack of sensitivity appears insufficient to explain the mismatch between gene expression and receptor activity. Moreover, the fact that DOR coupling is seen in the medial habenula and medulla, regions that have previously been shown to possess no detectable DOR-1 gene expression during development (Zhu *et al.*, 1998), provides further evidence of the overall sensitivity of the assay.

Low assay sensitivity, however, may explain the mismatch in other regions where levels of gene expression are considerably lower than the CPU, such as the midbrain, medial habenula, hypothalamus, and pons. At e12.5 and e13.5, MOR-1 expression in these regions begins at low levels (Zhu *et al.*, 1998), and at this stage, no MOR coupling can be detected, presumably because very little receptor protein is translated from the low levels of mRNA. By e15.5, MOR-1 gene expression has increased in these brain regions and approaches the high levels of expression seen in the CPU, medulla, and trigeminal ganglia (Zhu *et al.*, 1998). The appearance of detectable coupling at this stage most likely reflects the increased translation of protein resulting from higher levels of mRNA.

Alternatively, the lag between gene expression and receptor coupling may be due to a mismatch between levels of receptor protein and levels of mRNA. Subtype-specific receptor tissue section autoradiography and immunocytochemical data describing the relative abundance and anatomical distribution of opioid receptor protein during prenatal development is lacking, so it is difficult to assess whether a lack of translation is responsible for the mismatch; however, some comments can be made. Ligand binding studies indicate that MOR binding sites in brain homogenates are first detected at e12.5, whereas DOR binding sites are not detectable during the embryonic period (Rius *et al.*, 1991). Although these studies provide no anatomic localization of the opioid receptor protein, autoradiography experiments using [^{3}H]naloxone revealed opioid binding sites in the rat striatum at e14 (Kent *et al.*, 1981), which approximately equates to e12.5–e13 in the mouse. The relative correspondence between the ligand binding data in the mouse and the autoradiography in the rat with the GTPγ^{35}S binding experiments presented here provides further confidence in the sensitivity of the GTPγ^{35}S binding assays. In fact, the appearance of MOR coupling exactly coincides with the appearance of MOR binding sites detected in mice and naloxone binding sites in rats. In addition, although the ligand binding studies found no detectable DOR binding sites prior to birth, the present study has revealed DOR coupling as early as e17.5. Moreover, DOR coupling is seen in the medial habenula and medulla, regions that have previously been shown to possess no detectable DOR-1 gene expression during development (Zhu *et al.*, 1998). Taken together, these findings support the sensitivity of the assay and indicate that this receptor is positioned to impact embryonic development earlier than previously suspected based on ligand binding and gene expression studies.

Finally, the G-protein subtypes able to couple to the opioid receptors may not be expressed or translated at the stages when receptor mRNA is first seen. Although data describing the prenatal developmental pattern of G-protein expression is lacking, a recent study has explored the postnatal pattern of G-protein subunits by using quantitative immunoblotting (Ihnatovych *et al.*, 2002). This study revealed clearly detectable amounts of Gi and Go alpha subunits in the rat cortex, thalamus, and hippocampus in the p1 rat, and suggests that these G_α subtypes are likely found in other parts of the developing nervous system as well. Thus, it appears that the inability of the MOR and DOR to couple to G-proteins in certain brain regions at the later stages examined here is not due to a lack of available and appropriate G_α subtypes. This point is particularly relevant in the case of the trigeminal ganglia. As mentioned earlier, although high levels of MOR-1 gene expression are seen in this area beginning at e13.5, no MOR coupling is seen as late as p1, when Gi/Go is likely present. However, the possibility still remains that the absence of other cofactors necessary for G-protein activation is responsible for the delay in activity following the detection of gene expression found in the trigeminal ganglia and other brain regions.

Since GTPγ^{35}S binding assays only measure receptor coupling to Gi/Go G-proteins, the lack of coupling observed in the trigeminal ganglia, and other regions showing high levels of mRNA but no functional receptor activity, may not reflect a lack of coupling but rather interaction with other G alpha subtypes. Although the opioid receptors predominately interact with Gi/Go, several experiments attest to the ability of opioid receptor to couple to many other G-protein subtypes (Sanchez-Blazquez and Garzon, 1992, 1993; Sanchez-Blazquez *et al.*, 1993, 1995; Garzon *et al.*, 1994; Raffa *et al.*, 1994; Rossi *et al.*, 1995; Standifer *et al.*, 1996). Thus, transient activation of G-proteins other than Gi/Go by the MOR and DOR could allow functional activity and still explain the mismatch.

TABLE 1
Relative Amount of MOR Coupling during Development

	OB		*CPU*		*Hypo*		*MHb*		*MB*		*pons*		*Med*	
	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM
e12.5	—	—	+	46 ± 9	—	—	—	—	—	—	—	—	—	—
e13.5	—	—	+	46 ± 7	—	—	—	—	—	—	—	—	—	—
e15.5	—	—	++	64 ± 3	+	28 ± 3	+	47 ± 5	+	19 ± 2	+	28 ± 5	+	30 ± 3
e17.5	+	40 ± 3	++++	161 ± 31	++	73 ± 11	++	71 ± 2	++++	116 ± 21	+++	142 ± 21	++++	172 ± 5
p1	+	38 ± 7	++++	189 ± 19	++	76 ± 3	++	72 ± 20	+++	51 ± 5	++	100 ± 7	+++	117 ± 10

Note. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DAMGO for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. Each embryo of each stage was analyzed densitometrically by using the NIH Image software package. The density of GTPγ^{35}S binding was converted to nCi/g by using [^{14}C] standards enclosed in the film cassette. For each animal, in each brain region, nCi/g values from three agonist-stimulated sections were compared with three basal sections by using a *t* test to determine whether significant coupling was evident. Coupling was not considered present in a brain region unless significant coupling was seen in all embryos assessed at each stage. The nCi/g values are expressed as mean ± standard error of the mean, and the relative amounts scale is based on the following measurements: −, no significant coupling; +, <50 nCi/g [^{14}C]; ++, 51–100 nCi/g [^{14}C]; +++, 101–150 nCi/g [^{14}C]; ++++, 151–200 nCi/g [^{14}C]. OB, olfactory bulb; CPU caudate-putamen; Hypo, hypothalamus; MHb, medial habenula; MB, midbrain; Med, medulla.

Functional Implications

The present study has added a functional component to the studies of the opioid system during embryonic development. The results presented here describe the continued expansion of opioid receptor coupling from midgestation on and reveal that the changes in functional receptor activity do not always parallel changes in receptor gene expression or ligand binding. Thus, this study suggests that the interaction between opioid receptors, G-proteins, and other factors necessary for signaling is developmentally regulated. Similar regulation is seen in the cannabinoid 1 receptor (CB1) (Berrendero *et al.*, 1998). These studies showed that WIN-55,212–2 induced GTPγ^{35}S binding increased in the hippocampus in concert with increases in mRNA level from GD16 to GD18, while WIN-55,212–2 induced GTPγ^{35}S binding disappeared in the cortex despite an increase in mRNA in that same brain region. However, the scope of CB1 receptor regulation during development is difficult to assess because only a relatively limited period of development was investigated and the earliest stages of functional activity were not determined. By assessing opioid receptor function from their earliest detectable activation, we have shown that developmental regulation of GPCR is more widespread both temporally and spatially than suggested by the studies investigating the CB1 receptor.

The data outlined here also provide additional evidence that both the MOR and DOR can contribute to the adverse outcomes that follow *in utero* exposure to opiates. It has

TABLE 2
Relative Amount of DOR Coupling during Development

	OB		*CPU*		*Hypo*		*MHb*		*MB*		*pons*		*Med*	
	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM	Rel Amt	nCi/g SEM
e12.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
e13.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
e15.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
e17.5	—	—	—	—	++	55 ± 4	+	44 ± 20	—	—	++	80 ± 5	++	72 ± 5
p1	—	—	+	44 ± 12	+	44 ± 2	++	98 ± 23	—	—	++	74 ± 12	++	53 ± 1

Note. Sections were incubated with 2 mM GDP for 15 min, then with 0.04 nM GTPγ^{35}S, 2 mM GDP, and 3 μM DPDPE for 2 h. Basal GTPγ^{35}S binding was determined in the absence of agonist. Each embryo of each stage was analyzed densitometrically by using the NIH Image software package. The density of GTPγ^{35}S binding was converted to nCi/g by using [^{14}C] standards enclosed in the film cassette. For each animal, in each brain region, nCi/g values from three agonist-stimulated sections were compared with three basal sections by using a *t* test to determine whether significant coupling was evident. Coupling was not considered present in a brain region unless significant coupling was seen in all embryos assessed at each stage. The nCi/g values are expressed as mean ± standard error of the mean, and the relative amounts scale is based on the following measurements: −, no significant coupling; +, <50 nCi/g [^{14}C]; ++, 51–100 nCi/g [^{14}C]; +++, 101–150 nCi/g [^{14}C]; ++++, 151–200 nCi/g [^{14}C]. OB, olfactory bulb; CPU caudate-putamen; Hypo, hypothalamus; MHb, medial habenula; MB, midbrain; Med, medulla.

TABLE 3
Mismatch between Opioid Receptor Gene Expression and Receptor Coupling

Region	Begin μ gene expression	Begin μ coupling	Begin δ gene expression	Begin δ coupling
CPU	E11.5	E12.5	E17.5	P1
Midbrain	E12.5	E15.5	—	—
M. Habenula	E13.5	E15.5	—	E17.5
Hypothalamus	E13.5	E15.5	E13.5	E17.5
Pons	E12.5	E15.5	E13.5	E17.5
Trig. Ganglia	E13.5	—	—	—
Medulla	E12.5	E15.5	—	E17.5
Olf. Bulb	E17.5	E17.5	E17.5	—

Note. This table compares the onset of MOR-1 and DOR-1 gene expression, taken from (Zhu *et al.*, 1998), and the onset of receptor activity (present study) in various brain regions. CPU, caudate-putamen; M. Habenula, medial habenula; Trig. Ganglia, trigeminal ganglia; Olf. Bulb, olfactory bulb.

been shown that *in utero* exposure to morphine desensitizes the MOR in several medullary brain regions involved in respiration (Matsuda and Olsen, 2001). Since exogenous opiate drugs and endogenous opioid peptides produce respiratory depression, it was proposed that the MOR uncoupling found in the medulla is responsible for the hyperventilation seen in opiate-exposed pups. The CPU, shown in the present study to exhibit both DOR and MOR coupling during development, is involved in many facets of motor behavior (Lucien and Crutcher, 1991). Thus, perturbation of normal opioid signaling in this region during development by exogenous opiates could be responsible for the motor deficits seen in both rodents (Zagon and McLaughlin, 1978; Zagon *et al.*, 1979a; McLaughlin *et al.*, 1997a) and humans (Bunikowski *et al.*, 1998) exposed to opiates *in utero*. Further investigation is warranted to determine whether alterations in opioid receptor coupling in these and other regions discussed here are involved in the adverse effects of pre- and postnatal opiate exposure.

In conclusion, the results of this study outline for the first time the prenatal developmental pattern of opioid receptor activation, revealing the spatial and temporal patterns in which the necessary factors are assembled that allow the MOR and DOR to couple to G-proteins. Since receptor activity in some cases significantly lags behind the first appearance of opioid receptor mRNA, these findings suggest an intricate interaction between receptor expression, translation, and the availability of G-proteins and other signaling cofactors to produce functional signaling systems during development. Moreover, the demonstration of receptor activity during gestation indicates that the lack of a developmental phenotype in opioid receptor knockout mice is not due to a lack of receptor function during development. Instead, both the MOR and DOR have the capacity to influence nervous system function during both normal development and after exposure to exogenous opiate drugs.

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