Sandra Paula da Costa Pinto da Silva Rebelo e Sousa

ROLE OF THE TRANSCRIPTION FACTOR DRG11 IN THE EMBRYONIC DEVELOPMENT OF THE NOCICEPTIVE SYSTEM

Porto, 2010

DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR
APRESENTADA À FACULDADE DE MEDICINA
DA UNIVERSIDADE DO PORTO

Orientação do Professora Doutora Deolinda Maria Alves de Lima Teixeira
Co-orientação do Professor Doutor David Anderson

Sandra Paula da Costa Pinto da Silva Rebelo e Sousa

ROLE OF THE TRANSCRIPTION FACTOR DRG11 IN THE EMBRYONIC DEVELOPMENT OF THE NOCICEPTIVE SYSTEM

Artigo 48o, § 3o
"A Faculdade não responde pelas doutrinas expendidas na dissertação"
(Regulamento da Faculdade de Medicina do Porto, Decreto-Lei nº 19 337 de 29 de Janeiro de 1931)

CORPO CATEDRÁTICO
DA FACULDADE DE MEDICINA DO PORTO

Professores Efectivos

Alberto Manuel Barros da Silva
Altamiro Manuel Rodrigues Costa Pereira
Álvaro Jerónimo Leal Machado de Aguiar
António Carlos Freitas Ribeiro Saraiva
Daniel Filipe Lima Moura
Deolinda Maria Valente Alves Lima Teixeira
Francisco Fernando Rocha Gonçalves
Isabel Maria Amorim Pereira Ramos
João Francisco Montenegro Andrade Lima Bernardes
Jorge Manuel Mergulhão Castro Tavares
José Agostinho Marques Lopes
José Carlos Neves da Cunha Areias
José Eduardo Torres Eckenroth Guimarães
José Henrique Dias Pinto de Barros
José Manuel Lopes Teixeira Amarante
José Manuel Pereira Dias de Castro Lopes
Manuel Alberto Coimbra Sobrinho Simões
Manuel António Caldeira Pais Clemente
Manuel Jesus Falcão Pestana Vasconcelos
Maria Amélia Duarte Ferreira
Maria Dulce Cordeiro Madeira
Maria Fátima Machado Henriques Carneiro
Maria Leonor Martins Soares David
Patrício Manuel Vieira Araújo Soares Silva
Rui Manuel Almeida Mota Cardoso
Rui Manuel Lopes Nunes

Professores Jubilados ou Aposentados

Abel José Sampaio da Costa Tavares
Abel Vitorino Trigo Cabral
Alexandre Alberto Guerra Sousa Pinto
Amândio Gomes Sampaio Tavares
António Augusto Lopes Vaz
Antonio Carvalho Almeida Coimbra
António Fernandes da Fonseca
António Fernandes Oliveira Barbosa Ribeiro Braga
António Germano Pina Silva Leal
António José Pacheco Palha
António Luís Tomé da Rocha Ribeiro
António Manuel Sampaio de Araújo Teixeira
Belmiro dos Santos Patrício
Cândido Alves Hipólito Reis
Carlos Rodrigo Magalhães Ramalhão
Cassiano Pena de Abreu e Lima
Daniel Santos Pinto Serrão
Eduardo Jorge Cunha Rodrigues Pereira
Fernando de Carvalho Cerqueira Magro Ferreira
Fernando Tavarela Veloso
Francisco de Sousa Lé
Henrique José Ferreira Gonçalves Lecour de Menezes
Joaquim Germano Pinto Machado Correia da Silva
José Augusto Fleming Torrinha
José Carvalho de Oliveira
José Fernando Barros Castro Correia
José Luís Medina Vieira
José Manuel Costa Mesquita Guimarães
Levi Eugénio Ribeiro Guerra
Luís Alberto Martins Gomes de Almeida
Manuel Augusto Cardoso de Oliveira
Manuel Machado Rodrigues Gomes
Manuel Maria Paula Barbosa
Maria da Conceição Fernandes Marques Magalhães
Maria Isabel Amorim de Azevedo
Mário José Cerqueira Gomes Braga
Serafim Correia Pinto Guimarães
Valdemar Miguel Botelho dos Santos Cardoso
Walter Friedrich Alfred Osswald

JÚRI NOMEADO
PARA A PROVA DE DOUTORAMENTO

Presidente:

Reitor da Universidade do Porto

Vogais:

Doutora Deolinda Maria Valente Alves Lima Teixeira, professora catedrática da Faculdade de Medicina da Universidade do Porto, e orientadora da tese;

Doutor Quifu Ma, professor associado of Harvard Medical School;

Doutora Alexandra Matias Pereira da Cunha Coelho de Macedo, professora associada convidada da Faculdade de Medicina da Universidade do Porto;

Doutor Vasco Miguel Clara Lopes Galhardo, professor auxiliar da Faculdade de Medicina da Universidade do Porto;

Doutora Isabel Maria Mestre Marques Palmeirim de Alfarra Esteves, professora auxiliar da Universidade do Algarve;

Doutor Paulo Jorge Sousa Nunes Pereira, investigador do Instituto de Biologia Molecular e Celular do Porto.

À memória do Professor Doutor Manuel Miranda Guimarães

À Professora Doutora Deolinda Maria Alves de Lima Teixeira

Aos meus Pais e Irmão

Ao Miguel, Inês e Joana

PREFÁCIO

Finalmente! Não posso esconder o alívio que sinto ao escrever estas linhas pois fazem parte daquele que é o último texto que introduzo a esta dissertação antes de a dar como concluída. Passaram-se muitos anos desde o dia em que fui aceite como aluna de doutoramento da Faculdade de Medicina do Porto. Mais do que aqueles que gostaria que tivessem passado, mas foi o preço que tive de pagar por ter escolhido doutorar-me numa área, a do Desenvolvimento Embrionário, que até então nunca tinha sido explorada pela equipa de investigação dedicada ao estudo da dor chefiada pelo Professor Doutor Antonio Coimbra e mais tarde pela Professora Doutora Deolinda Lima. O desenvolvimento embrionário é uma área fascinante e desde os tempos de liceu que fiquei seduzida com as questões científicas e filosóficas subjacentes a esta área do conhecimento. Confesso, no entanto, que no remoto Junho de 1994, enquanto aluna do 5º ano de Ciências Farmacêuticas, estava muito longe de imaginar que um dia esta seria a minha área de especialização. Estava a preparar-me para fazer o último exame do curso (e aquele que julgava ser o último da minha vida … mal imaginava nos tantos que me esperavam em Medicina) quando recebi um convite surpreendente, por parte de uma Assistente que tinha sido minha professora de Farmacologia no 3º ano do curso, a Drª Sílvia Cunha, para participar num projecto de investigação que estava a desenvolver sob orientação da Professora Doutora Deolinda Lima. Foi de facto pela sua mão que entrei no mundo da investigação e pela porta do Instituto de Histologia e Embriologia da Faculdade de Medicina da Universidade do Porto. Agradeço-lhe o convite endereçado, o muito que me ensinou, a amizade que fomos desenvolvendo nos anos que juntas trabalhamos e a oportunidade de me apresentar a Professora Doutora Deolinda Lima. O entusiasmo e a paixão pela investigação transmitidas pela Professora Doutora Deolinda Lima, aquando do nosso primeiro encontro, juntamente com o clima acolhedor em que fui recebida, foram de tal modo contagiantes que não tive dúvidas que era consigo que queria trabalhar. Esta tese representa o esforço, dedicação, entusiasmo, rigor, empenho, dinamismo, determinação que são típicos da sua personalidade e que sabe transmitir aos seus discípulos. Estou-lhe profundamente agradecida e sensibilizada pela forma como sempre me apoiou e guiou. Não foi apenas uma orientadora, acima de tudo uma grande amiga. Ao Professor Doutor David Anderson, agradeço-lhe o privilégio que me concedeu, ao receber-me no seu laboratório, na *Division of Biology*, CalTech, EUA. Dotado de uma invulgar inteligência e rigor científico, acolheu-me calorosamente no seu grupo e deu-me a oportunidade de crescer cientificamente. Ao Professor Doutor Zhoufeng Chen, agradeço o facto de me ter concedido a honra de consigo partilhar a análise fenótipica do ratinho Drg11 *knockout* e que culminou no percurso escolhido para desenvolver esta dissertação. O seu incentivo e sentido de humor foram preciosos. Ao Professor Carlos Reguenga, desejo exprimir o meu mais sincero agradecimento por todo o dinamismo e conhecimento que soube imprimir quando se integrou nosso

pequeno grupo do Desenvolvimento. A presente dissertação beneficiou imenso com todo o seu contributo. À Doutora Liliana Osório, Dr. Carlos Pereira e Dra. Claúdia Lopes estou particularmente reconhecida por toda a colaboração e amizade dedicada enquanto bolseiros dos projectos aprovados pela FCT que financiaram os estudos conducentes à presente dissertação. Às Dras Mariana Matos e Isabel Regadas pelo seu contributo e alegria. À Professora Doutora Dulce Madeira e Professor Doutor Manuel Paula Barbosa o meu sincero reconhecimento por toda a ajuda prestada sobre métodos estereológicos que foi inestimável para um dos artigos aqui apresentados. Ao Professor Doutor Filipe Monteiro gostaria de agradecer a sua disponibilidade e precioso espírito crítico. Ao Professor Doutor António Coimbra, o meu sincero agradecimento por ter contribuído para a minha ida para os EUA, fiquei muito sensibilizada por o ver tão entusiasmado com a escolha do desenvolvimento embrionário como área do meu doutoramento. Aos Professores Doutores Claudio Sunkel e Elsa Bronze-da-Rocha, agradeço o estágio em técnicas de biologia molecular que me ofereceram antes da minha ida para os EUA. À Professora Doutora Maria da Conceição Magalhães expresso a minha gratidão, agradecendo todas as palavras de ânimo que me soube dar e ainda dá. Ao Professor Doutor José Castro Lopes, agradeço toda a amizade e apoio com que sempre me brindou, assim como a disponibilidade e espírito crítico que demonstrou quando solicitado. Aos Professores Doutores Duarte Pignatelli e Francisco Cruz, companheiros de antigo gabinete, quero expressar a minha admiração pela vossa boa disposição. Às Professoras Doutoras Fani Neto, Delminda Neves, Ana Charrua e Célia Cruz, o meu especial obrigada por toda a ajuda e recomendações/sugestões de quem já teve que passar pelo processo de escrita de dissertação. À Professora Doutora Isaura Tavares, gostava de reconhecer a honra que me concedeu ao coorientar informalmente a tese de mestrado integrado em medicina do Nuno Gonçalves. Ao Professor Doutor Henrique Almeida gostaria de agradecer os bons momentos que juntos passamos a trabalhar nos Mestrados de Medicina e Oncologia Molecular, serviram para aliviar o stress e foram retemperadores para o cérebro. Às Dras Joana Gomes e Clara Monteiro, amigas especiais, obrigada pelo vosso ombro amigo e excelentes momentos de "destilação". Um especial obrigado aos anos de excelente convívio e discussões científicas partilhadas com a Professora Doutora Ana Rita Castro. A todos os restantes elementos dos corpos docentes e de investigação do Serviço de Biologia Celular e Molecular e do Instituto de Histologia e Embriologia, agradeço o convívio enriquecedor e agradável com que sempre contei.

Um agradecimento muito sentido a todos os meus alunos que contribuíram de uma forma muito particular para a minha sanidade mental. Obrigada por todos os momentos joviais que juntos partilhamos. Foi um prazer poder participar de alguma forma na vossa formação enquanto excelentes futuros e presentes médicos.

Na Division of Biology, CalTech, gostaria de agradecer a todos os que contribuíram para que a minha estadia fosse tão agradável e aos preciosos ensinamentos, muito em particular aos Doutores Sherry Perez, Sean Morrison, Quifu Ma, Mariela Zirlinger, Sebastian Gerety, Emma Dormond, Amy Greenwood and Liching Lo. À Gabriele Mosconi, a Lab Manager mais eficiente do mundo, agradeço toda a amizade e preocupação durante a minha estadia no Anderson Lab.

Reconheço também o grande valor de todo o corpo técnico do Instituto de Histologia e Embriologia gostaria de agradecer a boa vontade, colaboração e preciosos ensinamentos com que sempre contei, muito em particular à Dª Alice Neves, Dª Maria Amélia Ferreira e Dª Elisa Nova no apoio laboratorial, ao Sr. Fernando Pinto no apoio do biotério e das Srs.ª D. Maria Teresa Laranjeira, Elisabete Ferreira, Raquel Madanços, no trabalho de secretariado. À Ana Tavares, quero realçar a amizade que sempre me dedicou.

A todas as pessoas responsáveis pela manutenção da colónia de ratinhos Drg11 knockout do Biotério do IBMC agradeço toda a dedicação e esforço em manter os animais nas melhores condições possíveis e gestão da colónia, muito em particular à Drª Luísa Guardão, Drª Isabel Carvalho, Isabel Duarte e Maria de Fátima Martins.

A presente tese de dissertação foi possível devido à concessão de bolsas de apoio à minha estadia no CalTech por parte da Fundação Calouste Gulbenkian e Fundação Luso-Americana (FLAD), assim com à concessão de uma Bolsa de Doutoramento pela Fundação para a Ciência e Tecnologia (PRAXIS XXI/BD/11519/97).

Ao Professor Doutor Vasco Galhardo gostaria agradecer de forma personalizada. A tua inteligência, amizade e incondicional apoio são uma bênção e motivo de orgulho. Sou tão sortuda de te ter como amigo. Tens o dom de tornar simples aquilo que por vezes é complicado, obrigada por tudo!

Ao Pedro Augusto, Isabel Reimão, Marta Drumond e Joana Queiróz Machado obrigada por terem sempre uma palavra de incentivo e de confiança.

É com imensa saudade que gostaria de reconhecer toda a amizade, confiança e reconhecimento prestados pelo Professor Doutor Manuel Miranda Magalhães, que um dia se lembrou de mim para engrossar o corpo docente da Biologia Celular e Molecular. Relembro comovida da vontade que manifestou em ver-me doutorada em vida. Não fui capaz de lhe realizar o desejo mas gostava dedicar à sua memória esta tese.

Aos meus pais, quero deixar expresso o meu reconhecimento pelo apoio incondicional, paciência, confiança, coragem, boa disposição e amor que sempre transmitiram. Imagino a preocupação que sentiram durante a minha ausência nos EUA mas souberam heroicamente guardá-la e aliviar-me as saudades. Não tenho palavras para vos agradecer e exprimir o quanto são importantes na minha vida, tudo o que sou a vocês devo. Obrigada por estarem sempre presentes e serem quem são.

Ao meu querido irmão, agradeço todo o incentivo, amizade e cumplicidade incondicionais, as palavras sempre apropriadas e revigorantes para os momentos de desânimo que senti ao longo deste processo. É um privilégio ter-te como irmão.

À Inês e Joana, quero agradecer-lhes a oportunidade que me ofereceram de vivenciar o desenvolvimento embrionário de outra perspectiva. Souberam ser uns embriões fortes e deixaram-me trabalhar com o mesmo vigor até ao último dia de gestação. Foi um prazer ter a vossa companhia na escrita da discussão de dois dos artigos aqui apresentados.

Por fim, quero expressar o meu mais sentido reconhecimento à pessoa que mais "sofreu" com toda esta tese. O Miguel teve que sobreviver às minhas ausências, ao desgaste e sacrifício implícito pela minha escolha profissional, que levou a que tivéssemos de adiar muitos projectos. Apesar de tudo, mantiveste-te sempre a meu lado, fiel companheiro dos bons e maus momentos. Quero que saibas que te admiro imenso e que me sinto muito abençoada por te ter como marido e pai das nossas meninas.

Em obediência ao disposto no Decreto-Lei nº 388/70, Artigo 8o, parágrafo 2, declaro que efectuei o planeamento e execução das experiências, observação e análise de resultados e participei activamente na redacção de todas as publicações que fazem parte integrante desta dissertação:

. I Rebelo S, Reguenga C, Osório L, Pereira C, Lopes C, Lima D (2007) DRG11 immunohistochemical expression during embryonic development in the mouse. Dev. Dyn. 236: 2653-2660.

. II Chen ZF, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ (2001) The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. Neuron 31: 59-73.

. III Rebelo S, Chen ZF, Anderson DJ, Lima D (2006) Involvement of DRG11 in the development of the primary afferent nociceptive system. Mol. Cell Neurosci. 33: 236-246.

. IV Rebelo S, Reguenga C, Lopes C, Lima D (2010) Prrxl1 is required for the generation of a subset of nociceptive glutamatergic superficial spinal dorsal horn neurons. Dev. Dyn. 239: 1684-1694.

. V Rebelo S, Lopes C, Lima D, Reguenga C (2009) Expression of a Prrxl1 alternative splice variant during the development of the mouse nociceptive system. Int. J. Dev. Biol. 53: 1089-1095.

A reprodução destas publicações foi feita com autorização das respectivas editoras.

ÍNDICE

1

INTRODUCTION

Pain is described by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Various stimuli, classically grouped as high threshold mechanical, thermal or chemical may cause pain perception.

Pain can be categorized in many different forms depending on the origin and nature of the triggering input and its intensity and duration. Of particular relevance is the division into acute (or physiological) and chronic pain (for review see Portenoy and Kanner, 1996). The latter can be subdivided into inflammatory and neuropathic pain (for review see Cervero and Laird, 1996), depending on its origin either on nociceptive stimulation of peripheral tissues or on lesionning of the nervous system, respectively.

THE NOCICEPTIVE SYSTEM

Pain is normally triggered when noxious stimuli of particular qualities are encoded by nociceptors (for review see Sherrington, 1906). Nociceptors consist on a specialized class of primary afferent sensory neurons located in the cranial and dorsal root ganglia that respond with a sole discharge to high threshold stimulation (Burgess and Perl, 1967). They are commonly divided in two classes: Aδ-fiber nociceptors and C-fiber nociceptors. Aδ-fiber nociceptors have fast-conducting, lightly myelinated axons and a broad cell body size spectrum (Lawson, 1992). They mediate sharp, pricking quality pain and are activated more efficiently by strong mechanical pressure and extreme heat. C-fiber nociceptors have slow-conducting, unmyelinated axons and small-diameter cell bodies (McCarthy and Lawson 1990; Lawson et al., 1996). They mediate burning quality pain and are activated by a variety of high-intensity mechanical, thermal, and chemical stimuli, therefore being commonly called polymodal (McCarthy and Lawson 1990; Lawson et al., 1996).

Nociceptors differ in neurotransmitter content and receptor and ion channel expression. They are commonly divided in two classes: peptidergic neurons, which contain substance P (SP) or calcitonin gene-related peptide (CGRP), express TrkA receptors and are NGF-responsive (Averill et al., 1995; Michael et al., 1997), and non-peptidergic neurons, which exhibit fluoride-resistant acid phosphatase (FRAP) and thiamine monophosphatase (TMP) activity (Silvermann and Kruger, 1990), bind to the lectin *Griffonia simplicifolia* (IB4) (Nagy and Hunt, 1982; Streit et al., 1986; Alvarez et al., 1991) and are GDNF-responsive (Moliver et al., 1997b; Bennett et al., 1998). Nociceptors express several transient receptor potential cation channels (TRPs) (for review see Vriens et al., 2009) such as TRPV1 (Caterina et al., 1997; Tominaga et al., 1998), TRPV2 (Caterina et al., 1999), TRPV3 (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002), TRPV4 (Schumacher et al., 2000; Guler et al., 2002), TRPA1 (Jaquemar et al., 1999; Story et al., 2003), TRPM8 (McKemy et al., 2002; Peier et al., 2002a), the purinergic receptor P2X3 (Chen et al., 1995; Lewis et al., 1995; Cook et al., 1997; for review see Wirkner et al., 2007) and Mas-related G-protein-coupled receptors (Mrgprs) (Dong et al., 2001; Lembo et al., 2002). From the large variety of Na^+ channel subunits (Nav) present in sensory fibers, Nav1.7, Nav1.8 and Nav1.9 are expressed preferentially in small DRG neurons, suggesting a possible role in nociception (Akopian et al., 1999; Dib-Hajj et al., 1998; Fang et al. 2002; Djouhri et al., 2003). The dorsal root acid sensing ion channel (DRASIC/ASIC3) (Waldmann et al., 1997) was shown to be present in peptidergic neurons (Price et al., 2001).

Nociceptors project to the dorsal horn of the spinal cord or to cranial sensory nuclei, where they impinge upon postsynaptic second-order neurons. They enter the spinal cord through the lateral division of the dorsal root to form the Lissauer tract, where they give rise to ascending and descending branches that extend for one to three segments (for review see Fyffe, 1992). Both Aδ and C primary afferents terminate in the most superficial laminae of the spinal cord dorsal horn. Peptidergic neurons project to lamina I and outer lamina II, while non-peptidergic neurons project to inner lamina II. Aδ primary afferents also project more deeply to terminate in lamina V (for review see Fyffe, 1992).

Information processed at the spinal level is conveyed to supraspinal centers via nociceptive-specific and wide dynamic range projection neurons. Projection neurons are located in laminae I and IV-V. Around 80% of lamina I projection neurons express the neurokinin 1 (NK1) receptor (Manthy et al., 1997; Doyle and Hunt, 1999; Todd, 2002).

Spinal projection neurons are feedback modulated, either directly or through local circuit neurons, upon activation of inhibitory and facilitatory descending pathways originated in multiple brain areas (Manthy et al., 1997; Stone et al., 1998; Stewart and Maxwell, 2000; Suzuki et al., 2002; Olave and Maxwell, 2003; for review see Lima and Almeida, 2002; Gebhart, 2004; Ossipov et al., 2010). Their final output is dependent on the interaction of various supraspinal and spinal neurotransmitter systems that are subjected to adjustment and plasticity, particularly under pathological conditions. Local systems include the fast inhibitory neurotransmitters γ-aminobutyric acid (GABA), which acts on ionotropic $GABA_A$ or G-protein-coupled metabotropic $GABA_B$ receptors, and glycine, which acts as a cotransmitter on ionotropic glycine receptors (Todd and McKenzie, 1989). Local inhibition is also mediated by endogenous opioids, such as met- and leu-enkephalin, β-endorphin, and dynorphin (Fields et al. 2006). Supraspinal descending modulatory systems use monoamines, such as noradrenalin, serotonin and dopamine (for review see Millan, 2002). Descending inhibition largely involves the spinal release of noradrenalin from brainstem nuclei such as the locus coeruleus and nucleus subcoerulus, which acts predominantly at the α2-adrenoceptor subclass to inhibit transmitter release from primary afferent terminals and suppress firing of projection neurons in the dorsal horn (for review see Millan, 2002). Neurotransmission of descending facilitation is much less studied. Serotoninergic pathways arising from the rostral ventromedial medulla (RVM) were initially shown to play a role in descending inhibition (Basbaum and Fields, 1984), but later revealed to exert bidirectional effects upon spinal nociception (Zhuo and Gebhart, 1991; Kovelowski et al., 2000; Buhler et al., 2005). Recent studies, using regional shRNA interference of neuronal tryptophan hydroxylase-2, showed that serotonin from spinal projecting RVM neurons is an important contributor to pain facilitation during the development of persistent pain (Wei et al., 2010).

A large variety of discrete brain areas are involved in pain perception (Apkarian et al., 2005), revealing the complexity of nociceptive processing in the central nervous system (for review see Tracey and Manthy, 2007). These areas are mainly located in the thalamus, hypothalamus, limbic system and cortex.

The development of chronic pain of either inflammatory or neuropathic nature is accompanied by dramatic changes at the various components of the nociceptive system. Such changes are based on multiple molecular alterations and result in the increase of receptive field size and in peripheral and central sensitization, with recruitment of unresponsive synapses and increased spontaneous and evoked firing (for review see Melzack and Wall, 1965; Treede et al., 1992; Cervero and Laird, 1996; Alvares and Fitzgerald, 1999; Hunt and Manthy, 2001; Julius and Basbaum, 2001). Spontaneous pain, which results from intermittent axonal depolarization and is characteristic of neuropathic pain, is accounted for by an increase in sodium channel expression (for review see Lai e at. 2004) and a decrease in potassium channel expression (Devor, 1983) in the DRG of the injured nerve. Hyperexcitability also develops in dorsal horn neurons, making both peripheral and spinal elements contributors to neuropathic pain (for review see Dubner and Ruda, 1992; Woolf and Salter, 2000). Inflammatory pain leads to altered activity of ion channels within affected sensory fibers, namely the purinergic P2X3 receptors and ASIC channels, (for review see Linley et al., 2010). Inflammatory mediators, which include bradykinin, SP, ATP, prostaglandins, growth factors, proteases, protons, nitric oxide (NO), cytokines and chemokines, among others (for review see McMahon et al., 2006), are capable of either sensitizing or directly exciting the peripheral terminals of nociceptive neurons (Shubayev and Myers, 2002; Schafers et al., 2003; Leinninger et al., 2004; for review see Anand, 2004).

Both aberrant neuronal activity and inflammatory mediators trigger several signaling pathways in primary afferent and dorsal horn sensory neurons, such as those involving protein kinases A and C,

calcium/calmodulin-dependent protein kinase and mitogen-activated protein kinases (MAPKs) (for review see Ji and Strichartzg, 2004). Moreover, activation of MAPKs in nonneuronal cells in the spinal cord, such as microglia and/or astrocytes, plays an important role in regulating excitability through the control of extracellular glutamate levels, and leads to the production of inflammatory mediators and sensitization of dorsal horn neurons (for review see Watkins et al., 2001a, b).

DEVELOPMENT OF THE NOCICEPTIVE SYSTEM

In order for the brain to accurately perceive noxious events, this complex nociceptive neuronal circuitry must be assembled with precision during embryonic development (for review see Gillespie and Walker, 2001; Julius and Basbaum, 2001). A comprehensive appraisal of the underlying mechanisms is essential for understanding how the nociceptive system functions and reacts to the establishment of chronic pain, and opens new frontiers for the development of more effective and specific pain therapies. In this respect, the neuronal circuitry linking the periphery with the central nervous system is of particular importance as a privileged site for therapeutical manipulation.

NEURAL TUBE FORMATION AND REGIONALIZATION

The anatomical outline of the mature central nervous system (CNS) is shaped first in the neuroepithelium and later in the early neural plate, as molecularly distinct progenitor regions are formed through the expression of unique combinations of specific transcription factors (for review see Lumsden and Krumlauf, 1996; Pituello, 1997; Rubenstein et al., 1998; Lee and Jessell, 1999; Shirasaki and Pfaff, 2002). In the mouse, neural folds begin to close at embryonic day 8 (E8) to form the neural tube (for review see Copp, 1990; Copp et al., 2003a, b; Greene and Copp, 2009). Between E8.5 and E10 (Serbedzija et al., 1990), a migratory cell population delaminates from the dorsal neural tube to form the neural crest cells (NCCs) (for review see LeDourin, 1980; LaBonne and Bronner-Fraser, 1999). Their migration occurs in chain-like structures to form the dorsal root ganglia (DRG) in a ventral to dorsal order, following a strict spatio-temporal signalling mechanism (Teillet et al., 1987; Lallier and Bronner-Fraser, 1988; Kasemeire-Kulesa, 2005). During migration and shortly after coalescing into a ganglion, NCCs are exposed to signals from the adjacent somites and neural tube (Liem et al., 1997; Martinsen and Bronner-Fraser, 1998; Garcia-Castro et al., 2002; for review see LaBonne and Bronner-Fraser, 1999) to become committed to a sensory neuronal fate. Then, they diversify into nociceptive, mechanoreceptive and proprioceptive sensory neurons.

The neural tube is patterned along its rostro-caudal and dorsal-ventral axes early in development (Jacobson and Gordon, 1976; Colas and Schoenwolf, 2001; for review see Schoenwolf and Smith, 1990; Diez del Corral and Storey, 2004). A series of constrictions appear in its wall, subdividing its anterior end into expanded vesicles, the forebrain, the midbrain and the hindbrain. The forebrain is later subdivided into telencephalon and diencephalon, and the hindbrain into the metencephalon and myelencephalon (for review see Gilbert, 2000). Initially, neural tube patterning is controlled by secreted extracellular signalling molecules that spread over variable distances, forming gradients across the neural tissue. These signals are spatio-temporally induced and define the specific transcriptional code that needs to be activated in distinct regions of the CNS for them to acquire their final structure (for review see Pituello, 1997; Harland, 2000; Tabata and Takei, 2004; Wilson and Houart, 2004).

Morphologically distinct subsets of cells can be recognized at predictable times and at precise positions in the neural tube (for review see Tanabe and Jessell, 1996). In the midline there is a narrow strip of non-neuronal cells forming dorsally the roof plate and ventrally the floor plate. Between these regions is the ventricular zone, which is formed by a pseudostratified epithelium of

proliferating neural progenitors (for review see Tanabe and Jessell, 1996). The position of progenitor cells along rostro-caudal and dorso-ventral axes is thought to influence their fate, but this is ultimately defined by the identity and concentration of exposing inductive signals. The acquisition of dorsal and ventral fates is dependent on short-range signals from non-neural ectoderm and notochord, respectively (for review see Tanabe and Jessell, 1996). Several TGF-β family members, including bone morphogenetic protein (BMP), are expressed in the roof plate and prospective neuroectoderm, and are critical in the specification of dorsal cell types (Basler et al., 1993; Liem et al., 1995; Liem et al., 1997; Lee et al., 1998; Wilson and Edlund, 2001; Timmer et al., 2002; Chesnutt et al., 2004; Win-Lee et al., 2004; for review see Lee and Jessell, 1999; Stern, 2001; Munoz-Sanjuan and Brivanlou, 2002; Chizhikov and Millen, 2005). Sonic hedgehog (Shh) signals from the notochord and first induces the formation of the floor plate to then promote the specification of ventral cell types (Marti et al., 1995; Roelink et al., 1995; Briscoe et al., 2001; Gritli-Linde et al., 2001 ; for review see Jessell and Dodd, 1990; Placzek, 1995;). Ventral neural tube patterning is also influenced by BMP signalling (Dale et al., 1999; McMahon et al., 1998; Liem et al, 2000). At a later time, ventral cell fate determination is dependent on Wnt ligands in conjunction with Shh signalling (Ulloa and Briscoe, 2007; Alvarez-Medina et al., 2008). Fibroblast growth factors (FGFs), produced by caudal mesoderm, are down-regulated before neural differentiation (for review see Wilson and Maden, 2005). In response to FGF down-regulation, retinoic acid (RA) is produced by the paraxial mesoderm and induces neural differentiation (Pierani et al., 1999; Wichterle et al., 2002; Diez del Corral et al., 2003; Novitch et al., 2003).

Fate mapping and molecular analyses of the spinal neural tube have depicted 11 neural progenitor domains, which produce distinct subpopulations of neurons in the dorsal (D1-D6) and ventral (po-p3, pMN) horns (for review see Caspary and Anderson, 2003; Helms and Johnson, 2003; Wilson and Maden, 2005). Progenitor domains (p0-p3, pMN) express differential combinatory codes of Class I and Class II homeodomain transcription factors and differentiate into distinct motor neuron subtypes (V0-V3, MN) in the ventral horn (for review see Wilson and Maden, 2005). Deep dorsal horn neurons are born after motor neurons from progenitor domains D1-D3, and superficial dorsal horn neurons, the last to mature, from progenitor domains D4-D6 (Altman and Bayer, 1984; for review see Wilson and Maden, 2005). While the mechanisms underlying the differentiation of spinal motor neurons are well understood, our knowledge on the molecular determinants of dorsal neuronal diversity is still limited.

SPECIFICATION OF THE PRIMARY SENSORY PATHWAY

DRG cells are born in successive waves (Frank and Sanes, 1991; Ma et al., 1999) that largely determinate their fate, connectivity, trophic factor dependence and function. In the mouse, cells from the first wave of neurogenesis are born between E9.5 and E11.5 and produce large-diameter-fiber TrkB and TrkC neurons, which mediate proprioceptive and mechanoceptive information, respectively (Lawson and Biscoe, 1979; Ma et al., 1999; for review see Marmigère and Ernfors, 2007). Cells from the second wave of neurogenesis are born between E10.5 and E13.5 and produce the majority of small-diameter-fiber TrkA-positive neurons, which mediate pain (Carr and Simpson, 1978; Lawson and Biscoe, 1979; Altman and Bayer, 1984; Kitao et al. 1996; Rifkin et al., 2000; Montelius et al., 2007; for review see Fariñas et al., 2002; Marmigère and Ernfors, 2007). Between E11.5-13.5, the boundary cap cells, a neural crest derivative, migrate along the central axonal projections of the already formed DRG neurons to colonize the DRG, thus feeding a secondary wave of peripheral neurogenesis (Maro et al., 2004). In the rat, at E15.5-16.5, a subpopulation of small-diameter-fiber neurons, probably the one that expresses CGRP, is produced (Kitao et al., 1996).

All these neurons require the bHLH transcription factors neurogenin 1 (Ngn1) and neurogenin 2 (Ngn2) early in specification (Perez et al., 1997; Fode et al., 1998, Ma et al., 1998; Ma, et al., 1999; Lo et al., 2002; for review see Anderson, 1999). Ngn2 is primarily needed for the generation of

TrkC$^+$ and TrkB$^+$ neurons, and Ngn1 for the generation of TrkA$^+$ neurons (Ma et al., 1999). Competitive interactions between these precursors may control the final proportions of different neuronal subtypes (for review see Fitzgerald, 2005).

Runx1 and Runx3, from the Runt-related (Runx) family of transcription factors (Levanon et al., 2001, 2002; Inoue et al., 2002; Marmigere et al., 2006; Chen et al., 2006a,b; Kramer et al., 2006; Nakamura et al., 2008) are required for further differentiation of sensory neurons. Runx3 differentiates the TrkC-positive, proprioceptor population from Ngn2-dependent neurons (Kramer et al., 2006; Marmigere et al., 2006) and regulates the spinal cord proprioceptor projection (Chen et al., 2006a). Runx1 differentiates subtypes of nociceptive neurons from the TrkA-positive population and regulates their projection to the dorsal horn (Yoshikawa et al., 2007; Chen et al., 2006b). Runx1 also acts postnatally on Ngn1-dependent neurons to suppress CGRP and TrkA expression, and thus differentiate a non-peptidergic subpopulation of DRG neurons that begins to express Ret and IB4 (Kramer et al., 2006, Molliver et al., 1997a, b).

In contrast to proprioceptors and nociceptors, little is known about the molecular mechanisms controlling the diversification of TrkB mechanosensitive neurons into distinct subtypes of low-threshold mechanoreceptors. Recently, it was shown that their differentiation depends on selective expression of the transcription factor MafA in combination with the Ret tyrosine kinase receptor and its coreceptor GFRα2 (Luo et al., 2007; 2009; Bourane et al., 2009).

The final numbers of DRG cells are determined by the balance between cell birth and programmed cell death, their survival being regulated by neurotrophic factors (for review see Kirstein and Fariñas, 2002). Peptidergic TrkA-positive neurons depend on nerve growth factor (NGF) (Silos-Santiago et al., 1995; Molliver et al., 1997a), while non-peptidergic TrkA-negative neurons (IB4-positive) depend on glial-derived neurotrophic factor (GDNF) (Molliver et al., 1997b; Bennett et al., 1996, 1998, 2000; Orozco et al., 2001; Zwick et al., 2002).

DEVELOPMENT OF PERIPHERAL AND CENTRAL PRIMARY AFFERENT CONNECTIONS

In the mouse, outgrowth of axons from the DRG to peripheral and central targets takes place at E10.5 (Ozaki and Snider, 1997). Innervation of the skin occurs in an organized manner, independently of motor innervation. The cutaneous nerve plexus is first build up by large-diameter A-fibers and immediately after by small-diameter C-fibers (Jackman and Fitzgerald, 2000) in a process that is regulated by neurotrophins (Kirsten and Farinas, 2002).

DRG axons arise at the dorsal root entry zone (DREZ) by day E10.5, but it takes 48 hours for them to extend collateral branches into the spinal gray matter (Ozaki and Snider, 1997). The physiological meaning of this waiting period, although documented in different species such as the rat, frog, cat and mice (Smith, 1983; Lee et al., 1988; Smith and Frank, 1988; Davis et al., 1989; Fitzgerald et al., 1991; Mirnics and Koerber, 1995), is not yet understood. At E13.5, a few primary afferent axons have entered the dorsal gray matter and course along the midline toward to the ventral spinal cord (Ozaki and Snider, 1997). By E15.5, axon projections to both the superficial and deep dorsal horn have developed (Ozaki and Snider, 1997). Each class of sensory axons projects directly to its target lamina, never branching into inappropriate laminae en route (Ozaki and Snider, 1997). Although the laminar architecture of the spinal cord is already established at E15.5 (for review see Jessell, 2000), the onset of terminal branching occurs later, at E18-19, after morphological and biochemical differentiation of distinct spinal cell groups is achieved (Fitzgerald, 1987; Mirnics and Koerber, 1995; Ozaki and Snider, 1997; Jackman and Fitzgerald, 2000).

As to the molecular mechanisms that guide DRG axons to their targets in the spinal cord, the role of chemorepulsive signals from the surrounding "nontarget" tissues, such as the dermamyotome, the notochord and the ventral spinal cord, is well established (Keynes et al., 1997; Nakamoto

and Shiga, 1998). Growing DRG axons express axonin-1, a GPI-anchored cell adhesion molecule of the immunoglobulin superfamily (Zuellig et al., 1992) that mediates notochord-derived chemorepulsion (Masuda et al., 2000, 2003). They also exhibit neuropilin-1 receptor, which is required for semaphorin 3A (Sema3A) signaling (Takagi et al., 1995; Kawakami et al., 1996; He and Tessier-Lavigne, 1997; Kitsukawa et al.,1997; Kolodkin et al., 1997; White and Behar, 2000; for review see Kolodkin and Ginty, 1997). Sema3A is a diffusible chemorepulsive from the ventral spinal cord that is involved in the regulation of the timing of DRG axonal entry into the spinal cord dorsal horn (Fu et al., 2000; Puschel et al., 1996; Shepherd et al., 1997; for review see Fujisawa and Kitsukawa, 1998), as well as in lamina-specific projection of NGF-dependent DRG axons (Messersmith et al., 1995).

Synaptic connections with primary afferent central targets in the spinal cord are established around birth (for review see Fitzgerald, 2005). Although data on the molecular mechanisms that guide proper connectivity are largely missing, it appears to depend on the same gene programs that direct subtype specification. In vertebrates, subtypes of primary sensory neurons have unique patterns of axon outgrowth and receptor expression immediately before target innervation (Guan et al., 2003). At birth, the primary afferent-spinal nociceptive pathway is established, but robust action potentials can not be evoked until the second postnatal week due to the low frequency of neurotransmitter release and immature state of the synapses (Fitzgerald and Jennings, 1999; Baccei et al., 2003).

SPECIFICATION OF SENSORY SPINAL NEURONS

The spinal dorsal horn hosts a large variety of sensory neurons specifically lodged in its different laminae (for review see Gillespie and Walker, 2001; Hunt and Mantyh, 2001; Julius and Basbaum, 2001). Several transcription factors have been uncovered as important to drive specification mechanisms and instruct neurons to fulfil their differentiation program (Muller et al., 2002; Qian et al., 2002; Zhou and Anderson, 2002; Cheng et al., 2004; Ding et al., 2004). Early born spinal dorsal horn neurons are generated at E10 from six progenitor domains (dp1-6), which express the proneural genes encoding the bHLH transcription factors Math1, Ngn1, Ngn2, Mash1 and Dbx2 (Gowan et al., 2001; for review see Caspary and Anderson, 2003; Helms and Johnson, 2003; Wilson and Maden, 2005; Lupo et al., 2006). Between E10-11.5, these progenitors give rise to six early-born dorsal neuronal populations (dI1-6), which will lodge in the deep dorsal horn (Gowan et al., 2001; Gross et al., 2002; Muller et al., 2002; Helms et al. 2005; for review see Lee and Jessell, 1999; Jessell, 2000; Chizhikov and Millen, 2005). Math 1-expressing progenitors give rise to dI1 interneurons (Helms and Johnson, 1998), Ngn1 and Ngn2 progenitors to dI2 interneurons (Gowan et al., 2001), Mash1 progenitors to dI3-5 interneurons (Qian et al., 2002) and Dbx2 progenitors to dI6 interneurons (Helms and Johnson, 2003). Early-born neurons can be subdivided into class A (dI1-3) and class B (dI4-6) neurons. Class A neurons arise from the dorsal alar plate, depend on roof plate signals and are Lbx1-independent (Liem et al., 1997; Lee et al., 1998, 2000; Wine-Lee et al., 2004); class B neurons arise from the ventral alar plate, are not dependent on roof plate signals and are Lbx1-dependent (Pierani et al., 2001; Gross et al., 2002; Muller et al., 2002; Cheng et al., 2004 ; for review see Matise et al., 2002). dI1-3 neurons are thought to be involved in proprioceptive processing (Bermingham et al., 2001; Gowan et al., 2001) and dI4-6 in nociceptive processing (Muller et al., 2002; for review see Goulding et al., 2002). Olig3 drives a marked increase in the number of dI3 cells in the presence of Mash1, and is therefore thought to impose, together with Mash1, the dI3 fate (Muller et al., 2005). Pax7, Dbx2 and Mash1 have been proposed as possible candidates for dI6 class-specific neuronal markers (Helms and Johnson, 2003; Muller et al, 2002).

At E12-14.5, a second neurogenic wave, derived from Mash1 expressing progenitors, produces two late-born neuronal populations, dILA and dILB. They arise in a salt-and-pepper pattern and migrate dorsally to form the superficial laminae of the dorsal horn (Gross et al., 2002; Muller et al.,

2002). dILA neurons differentiate into inhibitory neurons, which use GABA or glycine as fast transmitters. They require Ptf1a and Lbx1 for development and express the transcription factors Pax2 and Lhx1/5, as well as Gad1 (Glasgow et al., 2005; Cheng et al., 2004, 2005; Pillai et al., 2007). The expression of Lbx1, another homeobox gene, specifies default inhibitory GABAergic differentiation (Cheng et al., 2005). Gbx1 is also specifically expressed in dILA neurons, which, as development proceeds, differentiate into a subpopulation of GABAergic neurons (John et al., 2005). dILB neurons differentiate into excitatory neurons and use glutamate as neurotransmitter. They require Gsx1/2 for development, and express the transcription factors Tlx1/3 and Lmx1b, as well as vGlut2 (Gross et al., 2002; Muller et al., 2002, Cheng et al., 2004, 2005; Glasgow et al., 2005; Brohl et al., 2008; Xu et al., 2008). Tlx-class homeobox genes are determinant for the establishment of an excitatory glutamatergic nature (Cheng et al., 2004).

Between E18-18.5 peptidergic dorsal horn neurons are already differentiated in the various subpopulations. dlLA derived inhibitory neurons express category A neuropetides, which include NPY, nociceptin, dynorphin and enkephalin (Marti et al., 1987; Todd and Spike, 1993; Polgar et al., 2006). dILB derived excitatory neurons express category B neuropetides, such as CCK, TAC1, GRP and PACAP (Brohl et al., 2008; Xu et al., 2008).

SPINAL CIRCUITRY ESTABLISHMENT AND MATURATION

Maturation and tuning of spinal nociceptive circuits critically depends on the development of excitatory and inhibitory neurotransmitter/receptor functioning in the neonatal dorsal horn (for review see Fitzgerald, 2005). This depends as much on primary afferents and spinal neurons as on neurons sending descending projections from multiple brainstem nuclei.

Spontaneous activity, appearing early during spinal development, is regulated by the expression pattern of ion channels in individual neurons (for review see Fitzgerald, 2005). It is thought to be crucial for expression of distinct neuronal phenotypes, axonal growth, initial set of synaptic connections and signalling processes (for review see Moody, 1998; Moody and Bosma, 2005; Spitzer, 2006). While emerging excitability of embryonic motoneurons has been widely investigated (for review see Barbeau, 1999; Bate, 1999) little is known about that of spinal dorsal horn neurons.

Spinal networking strongly depends on the activity of glycinergic/GABAergic neurons, whose action is excitatory until shortly before birth (for review see Sibilla and Ballerini, 2009). The interplay between the glycinergic and GABAergic components in the spinal cord is subjected to dynamic changes throughout development, where the "predominance" of one transmitter system over the other depends on the stage of spinal maturation. In the mouse spinal cord, glycine levels are higher than GABA levels, indicating that at this early age glycinergic interneurons are already abundant (Miranda-Contreras et al. 2002). A progressive additional increment in glycine contents takes place between E17 and postnatal day 3 due to the appearance of numerous glycinergic neurons (Miranda-Contreras et al. 2002). As to GABA contents, there is also a gradual increase between E14 and P3 (Miranda-Contreras et al. 2002). These results are in line with previous data indicating an increased of the GABAergic component in the embryonic rat spinal cord activity up to E20 (Wu et al., 1992). However, immediately before birth GABA-mediated excitation is replaced by synaptic inhibition. The large majority of GABAergic neurons are located in the dorsal horn.

Functional elimination of synaptic inputs plays an important role in shaping adult connectivity in many parts of the nervous system (Shatz, 1983; Katz and Shatz, 1996; Katz and Crowley, 2002; Kim and Kandler, 2003; for review see Kano and Hashimoto, 2009), but its role on determining synaptic connectivity in the spinal dorsal horn is unclear. In the mouse, during the first postnatal week, a massive loss of glycinergic synapses occurs, together with a similar, but less pronounced loss in GABAergic synapses (Miranda-Contreras et al. 2002).

Brainstem nuclei differentiate between E11 and E16 in the rat and present their final anatomical features by E18 (Altman and Bayer, 1984). Axons descend from the brainstem to the spinal cord long before birth (Cabana and Martin, 1984), but they do not extend collateral branches into the dorsal horn for some time (Gilbert and Stelzner, 1979; Fitzgerald and Koltzenburg, 1986). This late development, which appears to depend on afferent C-fiber activity, is thought to explain the delayed postnatal onset of functional descending inhibition (Cervero and Plenderleith, 1985). Electrical activation of the PAG does not produce analgesia until P21 (van Praag and Frenk, 1991) and stimulation of the dorsolateral funiculus cannot inhibit firing of dorsal horn neurons until P10 (Boucher et al., 1998; Fitzgeral and Koltzenburg, 1986). Descending fibers transection before P15 has less impact on spinal sensory circuits than it does later in life (Weber and Stelzner, 1977).

OBJECTIVES AND STUDY OUTLINE

Experimental data concerning the molecular mechanisms of development of the nervous system were scarce in the late nineties of the past century. At that time, however, mouse genetics had reached sufficient sophistication to allow the combination of molecular, embryological, biochemical and genetic approaches, which proved to be capable of revealing the principles that control the diversification and patterning of the vertebrate nervous system (Tanabe and Jessell, 1996). From then on, seminal studies have uncovered the basic mechanisms that govern neuronal differentiation at the ventral and dorsal spinal cord (reviewed above).

The acknowledgment that transcription factors coordinate several key biological processes in nervous system development points to a new way of thinking the development and plasticity of neuronal circuits. A set of transcription factors involved in the development of sensory neurons and their differentiation into excitatory and inhibitory populations was identified, but very scarce data were obtained on the molecular mechanisms that govern the development of the nociceptive system. Only one study by the group of David Anderson (Saito and collaborators, 1995) approached this issue by revealing a novel paired-like homeodomain transcription factor, Drg11 (recently renamed as Prrxl1), which is specifically expressed in small size DRG neurons and in the superficial spinal cord dorsal horn. Based on its early expression and particular location, Drg11 was regarded as possibly playing a role as a master regulator of differentiation of the spinal nociceptive circuit.

Following an old venture of unravelling the molecular processes that underlie the specification of the various categories of superficial dorsal horn neurons, a collaboration was set up with David Anderson aimed at functionally characterizing Drg11 as a putative determinant of the differentiation of the nociceptive system through the study of a Drg11 knockout mouse model. The resulting studies, which make up the bulk of the present thesis, were guided by the following objectives:

1) To determine whether Drg11 may extend its role to the cranial level

2) To determine whether Drg11 is involved in the development of the nociceptive system

3) To determine the specific role of Drg11 in the differentiation of nociceptive primary afferent and spinal neurons

4) To evaluate whether the differential involvement of Drg11 in DRG and spinal cord development is explained by the occurrence of Drg11 splice variants

The data collected during this study were published in the following five original papers.

In the first publication (Developmental Dynamics, vol. 236), systematic spatio-temporal immunohistochemical analysis of Drg11 expression in the entire peripheral and central mouse nervous system was carried out along embryonic development and postnatally. To accomplish this purpose, a polyclonal anti-Drg11 antibody was raised in rabbit against the C-terminal region.

The second publication (Neuron, vol. 31) analysed the phenotypic profile resulting from the deletion of the Drg11 gene (the two exons that correspond to the putative DNA binding region) in mice using homologous recombination in embryonic stem cells. Early developmental phenotypic

abnormalities in the DRG and spinal cord dorsal horn of *Drg11*$^{-/-}$ embryos were searched using *in situ* hibridization and Nissl staining. Persistent anatomical and molecular deficiencies in the adult DRG and spinal cord were also looked for. Nociceptive function was experimentally assessed by performing a battery of behavioural tests in adult mice.

In the third publication (Molecular Cell Neuroscience, vol. 33), the involvement of Drg11 in the development of primary afferent nociceptive neurons was addressed. The various subclasses of DRG neurons were quantified in wild-type and *Drg11*$^{-/-}$ embryos and adult mice by the use of stereological methods, and the extent to which the innervation of various peripheral tissues was affected by the deletion mutant was investigated.

The fourth publication (Developmental Dynamics, vol. 239) addressed the immunohistochemical expression of spinal Drg11 along development together with two other functionally related transcription factors, Tlx3 and Lmx1b, as a way of defining various subpopulations of spinal cord dorsal horn Drg11-dependent neurons. By the use of the Golgi-Rio Hortega silver impregnation method, the extent to which the loss of Drg11-dependent neurons in the Drg11 knockout mice affected the anatomy of the spinal cord dorsal horn was evaluated. The way in which noxious-evoked neuronal activation at the spinal level was affected was also investigated by immunohistochemical detection of c-fos induction.

In the fifth publication (International Journal of Developmental Biology, vol. 53), a Drg11 alternative splice variant was reported and its expression along development at the DRG and spinal levels characterized. Mouse Drg11 isoform mRNA sequences were obtained by Rapid Amplification cDNA Ends (RACE) analysis and the distribution of the splice isoform at different developmental ages was analysed by in situ hibridization and quantitative real-time PCR.

All experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) and the ethical guidelines for pain investigation in animals (Zimmerman, 1983).

Akopian AN, Souslova V, England S, Okuse K, Ogata N, Ure J, Smith A, Kerr BJ, McMahon SB, Boyce S, Hill R, Stanfa LC, Dickenson AH, Wood JN (1999). The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat. Neurosci. 2: 541-548.
Altman J, Bayer SA (1984). The development of rat spinal cord. Adv. Anat. Embryol. Cell Biol. 85: 1-168.
Alvarez FJ, Morris H, Priestley JV (1991). Sub-populations of smaller diameter trigeminal primary afferent neurons defined by expression of calcitonin gene-related peptide and the cell surface oligosaccharide recognized by monoclonal antibody LA4. J. Neurocytol. 20: 716-731.
Alvarez-Medina R, Cayuso J, Okubo T, Takada S, Martí E (2008). Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression. Development135: 237-247.
Alvares D, Fitzgerald M (1999) Building blocks of pain: the regulation of key molecules in spinal sensory neurones during development and following peripheral axotomy. Pain Suppl 6: S71-85.
Anand P (2004).Neurotrophic factors and their receptors in human sensory neuropathies. Prog. Brain Res 146: 477–492.
Anderson DJ (1999). Lineages and transcription factors in the specification of vertebrate primary sensory neurons. Curr. Opin Neurobiol. 9: 517-524.
Apkarian AV, Bushnell MC, Treede RD, Zubieta JK (2005). Human brain mechanisms of pain perception and regulation in health and disease. Eur. J. Pain 9: 463-484.
Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur. J. Neurosci. 7: 1484-1494.
Baccei ML, Bardoni R, Fitzgerald M (2003). Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. J. Physiol. 549: 231-242.
Basbaum AI, Fields HL (1984). Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu. Rev. Neurosci. 7: 309-338.
Basler K, Edlund T, Jessell TM, Yamada T (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGF beta family member. Cell 73: 687-702.
Barbeau H, McCrea DA, O'Donovan MJ, Rossignol S, Grill WM, Lemay MA (1999). Tapping into spinal circuits to restore motor function. Brain Res. Rev. 30: 27–51.
Bate M (1999). Development of motor behaviour. Curr. Opin. Neurobiol. 9: 670–675.
Bennett DLH, Averill S, Clary DO, Priestley JV, McMahon SB (1996). Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. Eur. J. Neurosci. 8: 2204-2208.
Bennett DLH, Boucher TJ, Armanini MP, Poulsen KT, Michael GJ, Priestley JV, Philips HS, McMahon SB, Shelton DL (2000). The glial cell line-derived neurotrophic factor family receptor components are differentially regulated within sensory neurons after nerve injury. J. Neurosci. 20: 427-437.
Bennett DLH, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998). A distinct subgroup of small DRG cels express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J. Neurosci. 18: 3059-3072.
Bermingham NA, Hassan BA, Wang VY, Fernandez M, Banfi S, Bellen HJ, Fritzsch B, Zoghbi HY (2001). Proprioceptor pathway development is dependent on Math1. Neuron30: 411-422.
Boucher T, Jennings E, Fitzgerald M (1998). The onset of diffuse noxious inhibitory controls in postnatal rat pups: a C-Fos study. Neurosci. Lett. 257: 9-12.
Bourane S, Garces A, Venteo S, Pattyn A, Hubert T, Fichard A, Puech S, Boukhaddaoui H, Baudet C, Takahashi S, Valmier J, Carroll P (2009). Low-threshold mechanoreceptor subtypes selectively express MafA and are specified by Ret signaling. Neuron 64: 857-870.
Burgess PR, Perl ER (1967). Myelinated afferent fibers responding specifically to noxious stimulation of the skin. J. Physiol. 190: 541-562.
Briscoe J, Chen Y, Jessell TM, Struhl G (2001). A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. Mol. Cell. 7: 1279-1291.
Bröhl D, Strehle M, Wende H, Hori K, Bormuth I, Nave KA, Müller T, Birchmeier C (2008). A transcriptional network coordinately determines transmitter and peptidergic fate in the dorsal spinal cord. Dev. Biol. 322: 381-393.
Buhler AV, Choi J, Proudfit HK, Gebhart GF (2005). Neurotensin activation of the NTR1 on spinally-projecting serotonergic neurons in the rostral ventromedial medulla is antinociceptive. Pain 114: 285-294.
Cabana T, Martin GF (1984). Developmental sequence in the origin of descending spinal pathways. Studies using retrograde transport techniques in the North American opossum (Didelphis virginiana). Brain Res. 317: 247-263.
Carr VM, Simpson SB Jr (1978). Proliferative and degenerative events in the early development of chick dorsal root ganglia. I. Normal development. J. Comp. Neurol. 182: 727-739.
Caspary T, Anderson KV (2003). Patterning cell types in the dorsal spinal cord: what the mouse mutants say. Nat. Rev. Neurosci. 4: 289-297.
Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389: 816-824.
Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398: 436-441.
Cervero F, Laird JM (1996). From acute to chronic pain: mechanisms and hypoyheses. Prog. Brain Res. 110: 3-15.
Cervero F, Plenderleith MB (1985). C-fibre excitation and tonic descending inhibition of dorsal horn neurones in adult rats treated at birth with capsaicin. J. Physiol. 365: 223-237.
Chen AI, Broom DC, Liu Y, de Nooij JC , Li Z, Cen C, Samad OA, Jessell TM, Woolf CJ, Ma Q (2006b). Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron 49: 365-377.
Chen AI, de Nooij JC, Jessell TM (2006a). Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. Neuron 49: 395-408.
Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995). A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377: 428-431.
Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, Arata S, Shirasawa S, Bouchard M, Luo P, Chen C-L, Busslinger M, Goulding M, Onimaru H, Ma Q (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat. Neurosci. 7:510-517.
Cheng L, Samad OA, Xu Y, Mizuguchi R, Luo P, Shirasawa S, Goulding M, Ma Q (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat. Neurosci. 8: 1510-1515.

Chesnutt C, Burrus LW, Brown AM, Niswander L (2004). Coordinate regulation of neural tube patterning and proliferation by TGFβ and WNT activity. Dev. Biol. 274: 334-347.
Chizhikov, VV, Millen, KJ (2005). Roof plate-dependent patterning of the vertebrate dorsal central nervous system. Dev. Biol. 277: 287-295.
Colas JF, Schoenwolf GC (2001). Towards a cellular and molecular understanding of neurulation. Dev. Dyn. 221: 117-145.
Cook SP, Vulchanova L, Hargreaves KM, Elde R, McCleskey EW (1997). Distinct ATP receptors on pain-sensing and stretch-sensing neurons. Nature 387: 505-508.
Copp AJ, Brook FA, Estibeiro JP, Shum AS, Cockroft DL (1990). The embryonic development of mammalian neural tube defects. Prog. Neurobiol. 35: 363-403.
Copp AJ, Greene ND, Murdoch JN (2003a). Disheyelled: linking convergent extension with neural tube closure. Trends Neurosci. 26: 453-455.
Copp AJ, Greene ND, Murdoch JN (2003b). The genetic basis of mammalian neurolation. Nat. Rev. Genet. 4: 784-793.
Dale K, Sattar N, Heemskerk J, Clarke JD, Placzek M, Dodd J (1999). Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin. Development 126: 397-408.
Davis BM, Frank E, Johnson FA, Scott SA (1989). Development of central projections of lumbosacral sensory neurons in the chick. J. Comp. Neurol. 279: 556-566.
Devor M (1983). Potassium channels moderate ectopic excitability of nerve-end neuromas in rats. Neurosci. Lett. 40: 181–186.
Dib-Hajj SD, Tyrrell L, Black JA, Waxman SG (1998). NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. Proc. Natl. Acad. Sci. U S A 95: 8963-8968.
Diez del Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey KG (2003). Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40: 65-79
Diez del Corral R, Storey KG (2004). Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. BioEssays 26: 857-869
Ding YQ, Yin J, Kania A, Zhao ZQ, Johnson RL, Chen ZF (2004). Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. Development 131: 3693-3703.
Djouhri L, Fang X, Okuse K, Wood JN, Berry CM, Lawson SN (2003). The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. J. Physiol. 550: 739-752.
Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ (2001). A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell 106: 619-632.
Doyle CA, Hunt SP (1999). Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a c-Fos study in rat. Neuroscience 89: 17-28.
Dubner R, Ruda MA (1992). Activity-dependent neuronal plasticity following tissue injury and inflammation. Trends Neurosci. 15: 96-103.
Fang X, Djouhri L, Black JA, Dib-Hajj SD, Waxman SG, Lawson SN (2002). The presence and role of the tetrodotoxin-resistant sodium channel Na(v)1.9 (NaN) in nociceptive primary afferent neurons. J. Neurosci 22: 7425-7433.
Fariñas I, Cano-Jaimez M, Bellmunt E, Soriano M (2002). Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res. Bull. 57: 809-816.
Fitzgerald M (2005). The development of nociceptive circuits. Nat. Rev. Neurosci. 6: 507-520.
Fitzgerald M (1987). The prenatal growth of fine diameter afferents into rat spinal cord - a transganglionic tracer study. J. Comp. Neurol. 261: 98-104.
Fitzgerald M, Jennings E (1999). The postnatal development of spinal sensory processing. Proc. Natl. Acad. Sci. U.S.A. 96: 7719-7722.
Fitzgerald M, Koltzenburg M (1986). The functional development of descending inhibitory pathways in the dorsolateral funiculus of the newborn rat spinal cord. Brain Res. 389: 261-270.
Fitzgerald M, Reynolds ML, Benowitz LI (1991). GAP-43 expression in the developing rat lumbar spinal cord. Neuroscience 41: 187-199.
Fode C, Gradwohl G, Morin X, Dierich A, LeMeur M, Goridis C, Guillemot F (1998). The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron 20: 483-494.
Frank E, Sanes JR (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis in vivo with a recombinant retrovirus. Development 111: 895-908.
Fu SY, Sharma K, Luo Y, Raper JA, Frank E (2000). SEMA3A regulates developing sensory projections in the chicken spinal cord. J. Neurobiol. 45: 227-236.
Fujisawa H, Kitsukawa T (1998). Receptors for collapsin/semaphorins. Curr. Opin. Neurobiol. 8: 587-592.
Fyffe REW. Laminar Organization of Primary afferent terminations in the mammalian spinal cord. Sensory neurons. Diversity, development and plasticity. Edited by S.A.Scott. OUP, New York.
García-Castro MI, Marcelle C, Bronner-Fraser M (2002). Ectodermal Wnt function as a neural crest inducer. Science 297: 848-851.
Gebhart GF (2004). Descending modulation of pain. Neurosci. Biobehav. Rev. 27: 729-737.
Gilbert M, Stelzner DJ (1979). The development of descending and dorsal root connections in the lumbosacral spinal cord of the postnatal rat. J. Comp. Neurol. 184: 821-838.
Gilbert SF (2000). The central nervous system and the epidermis. In: Sinauer Associatess (Ed), Development Biology, 6Th Ed., Sunderland, Massachusetts, pp. 379-410.
Gillespie PG, Walker RG (2001). Molecular basis of mechanosensory transduction. Nature 413: 194-202.
Glasgow SM, Henke RM, MacDonald RJ, Wright CVE, Jonhson JE (2005). Ptf1 determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. Development 132: 5461-5469.
Gowan K, Helms AW, Hunsaker TL, Collisson T, Ebert PJ, Odom R, Johnson JE (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron 31: 219-232.
Goulding M, Lanuza G, Sapir T, Narayan S (2002). The formation of sensorimotor circuits. Curr. Opin. Neurobiol. 12: 508-515.
Greene ND, Copp AJ (2009). Development of the vertebrate central nervous system: formation of the neural tube. Prenat. Diagn. 29: 303-311.
Gritli-Linde A, Lewis P, McMahon AP, Linde A (2001). The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. Dev. Biol. 236: 364-86.
Gross MK, Dottori M, Goulding M (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34: 535-549.
Guan W, Puthenveedu MA, Condic ML (2003). Sensory neuron subtypes have unique substratum preference and receptor expression before target innervation. J. Neurosci. 23: 1781-1791.
Güler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M (2002). Heat-evoked activation of the ion channel, TRPV4. J. Neurosci. 22: 6408-6414.

Harland R (2000). Neural induction. Curr. Opin. Genet. Dev. 10: 357-362.
He Z, Tessier-Lavigne M (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90: 739-751.
Helms AW, Johnson JE (2003). Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol. 13: 42-49.
Helms AW, Johnson JE (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. Development 125: 919-928.
Helms AW, Battiste J, Henke RM, Nakada Y, Simplicio N, Guillemot F, Johnson JE (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. Development 132: 2709-2 719.
Hunt SP, Mantyh PW (2001). The molecular dynamics of pain control. Nat. Rev. Neurosci. 2: 83-91.
Inoue K, Ozaki S, Shiga T, Ito K, Masuda T, Okado N, Iseda T, Kawaguchi S, Ogawa M, Bae SC, Yamashita N, Itohara S, Kudo N, Ito Y (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat. Neurosci. 5: 946-954.
Jackman A, Fitzgerald M (2000). Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. J. Comp. Neurol. 418: 281-298.
Jacobson AG, Gordon R (1976). Changes in the shape of the developing vertebrate nervous system analyzed experimentally, mathematically and by computer simulation. J. Exp. Zool. 197: 191-246.
Jaquemar D, Schenker T, Trueb B (1999). An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. J. Biol. Chem. 274: 7325-7333.
Jessell TM, Dodd J (1990). Floor plate-derived signals and the control of neural cell pattern in vertebrates. Harvey Lect. 86: 87-128.
Jessell TM (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. 1: 20-29.
Ji RR, Gereau RW 4th, Malcangio M, Strichartz GR (2009). MAP kinase and pain. Brain Res. Rev. 60: 135-48.
John A, Wildner H, Britsch S (2005). The homeodomain transcription factor Gbx1 identifies a subpopulation of late-born GABAergic interneurons in the developing dorsal spinal cord. Dev. Dyn. 234: 767-771.
Julius D, Basbaum AI (2001). Molecular mechanisms of nociception. Nature 413: 203-210.
Kano M, Hashimoto K (2009). Synapse elimination in the central nervous system. Curr. Opin. Neurobiol. 19: 154-161.
Kasemeier-Kulesa JC, Kulesa PM, Lefcort F (2005). Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. Development. 132: 235-245.
Katz LC, Crowley JC (2002). Development of cortical circuits: lessons from ocular dominance columns. Nat. Rev. Neurosci. 3: 34-42.
Katz LC, Shatz CJ (1996). Synaptic activity and the construction of cortical circuits. Science 274: 1133-1138.
Kawakami A, Kitsukawa T, Takagi S, Fujisawa H (1996). Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. J. Neurobiol. 29: 1-17.
Keynes R, Tannahill D, Morgenstern DA, Johnson AR, Cook GM, Pini A (1997). Surround repulsion of spinal sensory axons in higher vertebrate embryos. Neuron 18: 889-897.
Kim G, Kandler K (2003). Elimination and strengthening of glycinergic/GABAergic connections during tonotopic map formation. Nat. Neurosci. 6: 282-290.
Kirstein M, Fariñas I (2002). Sensing life: regulation of sensory neuron survival by neurotrophins. Cell. Mol. Life Sci. 59: 1787-1802.
Kitao Y, Robertson B, Kudo M, Grant G (1996). Neurogenesis of subpopulations of rat lumbar dorsal root ganglion neurons including neurons projecting to the dorsal column nuclei. J. Comp. Neurol. 371: 249-257.
Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in periph eral nerve projection in mice. Neuron 19: 995-1005.
Kolodkin AL, Ginty DD (1997). Steering clear of semaphorins: neuropilins sound the retreat. Neuron 19: 1159-1162.
Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD (1997). Neuropilin is a semaphorin III receptor. Cell 90: 753-762.
Kovelowski CJ, Ossipov MH, Sun H, Lai J, Malan TP, Porreca F. Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to maintain neuropathic pain in the rat. Pain 87: 265-273.
Kramer I, Sigrist M, de Nooij JC, Taniuchi I, Jessell TM, Arber S (2006). A role for Runx transcription factor signalling in dorsal root ganglion sensory neuron diversification. Neuron 49: 379-393.
LaBonne C, Bronner-Fraser M (1999). Molecular mechanisms of neural crest formation. Annu. Rev. Cell Dev. Biol. 15: 81-112.
Lai J, Porreca F, Hunter JC, Gold MS (2004). Voltage-gated sodium channels and hyperalgesia. Annu. Rev. Pharmacol.Toxicol. 44: 371–397.
Lallier TE, Bronner-Fraser M (1988). A spatial and temporal analysis of dorsal root and sympathetic ganglion formation in the avian embryo. Dev. Biol. 127: 99-112.
Lawson SN (1992). Morphological and biochemical cell types of sensory neurons. In Sensory neurons. Diversity, development and plasticity. Edited by SA Scott. OUP, New York. pp.27-59.
Lawson SN, Biscoe TJ (1979). Development of mouse dorsal root ganglia: an autoradiographic and quantitative study. J. Neurocytol. 8: 265-274.
Lawson SN, Crepps B, Buck H, Perl ER (1996). Correlation of CGRP-like immunoreactivity (CGRP-LI) with sensory receptor properties in dorsal root ganglion (DRG) neurons in guinea pigs. J. Physiol. 493P:P45.
Le Douarin N (1980). Migration and differentiation of neural crest cells. Curr. Top. Dev. Biol. 16: 31-85.
Levanon D, Bettoun D, Harris-Cerruti C, Woolf E, Negreanu V, Eilam R, Bernstein Y, Goldenberg D, Xiao C, Fliegauf M, Kremer E, Otto F, Brenner O, Lev-Tov A, Groner Y (2002). The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. EMBO J. 21: 3454-3463.
Levanon D, Brenner O, Negreanu V, Bettoun D, Woolf E, Eilam R, Lotem J, Gat U, Otto F, Speck N, Groner Y (2001). Spatial and temporal expression pattern of Runx3 (Aml2) and Runx1 (Aml1) indicates non-redundant functions during mouse embryogenesis. Mech. Dev. 109: 413-417.
Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995). Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377: 432-435.
Lee KJ, Dietrich P, Jessell TM (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. Nature 403: 734-740.
Lee KJ, Jessell TM (1999). The specification of dorsal cell fates in the vertebrate central nervous system. Annu. Rev. Neurosci. 22: 261-294.
Lee KJ, Mendelsohn M, Jessell TM (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev. 12: 3394-3407.
Leinninger GM, Vincent AM, Feldman EL (2004). The role of growth factors in diabetic peripheral neuropathy, J. Peripher. Nerv. Syst. 9: 26–53.
Lembo PM, Grazzini E, Groblewski T, O'Donnell D, Roy MO, Zhang J, Hoffert C, Cao J, Schmidt R, Pelletier M, Labarre M, Gosselin M, Fortin Y, Banville D, Shen SH, Ström P, Payza K, Dray A, Walker P, Ahmad S (2002). Proenkephalin A gene products activate a new family of sensory neuron--specific GPCRs. Nat. Neurosci. 5: 201-209.

Liem KF Jr, Jessell TM, Briscoe J (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. Development 127: 4855-4866.
Liem KF Jr, Tremmi G, Jessell TM (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91: 127-138.
Liem KF Jr, Tremmi G, Roelink H, Jessell TM (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 82: 969-979.
Lima D, Almeida A (2002). The medullary dorsal reticular nucleus as a pronociceptive centre of the pain control system. Prog. Neurobiol. 66: 81–108.
Linley JE, Rose K, Ooi L, Gamper N (2010). Understanding inflammatory pain: ion channels contributing to acute and chronic nociception. Pflugers Arch. 459: 657-669.
Lo L, Dormand E, Greenwood A, Anderson DJ (2002). Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development 129: 1553-1567.
Lumsden A, Krumlauf R (1996). Patterning the vertebrate neuraxis. Science 274: 1109-1115.
Luo W, Enomoto H, Rice FL, Milbrandt J, Ginty DD (2009). Molecular identification of rapidly adapting mechanoreceptors and their developmental dependence on ret signaling. Neuron 64: 841-856.
Luo W, Wickramasinghe SR, Savitt JM, Griffin JW, Dawson TM, Ginty DD (2007). A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. Neuron 54: 739-754.
Lupo G, Harris WA, Lewis KE (2006). Mechanisms of ventral patterning in the vertebrate nervous system. Nat. Rev. Neurosci. 7: 103-114.
Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20: 469-482.
Ma Q, Fode C, Guillemot F, Anderson DJ (1999). Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev. 13: 1717-1728.
Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA (1997). Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. Science 278: 275-279.
Marmigère F, Ernfors P (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. Nat. Rev. Neurosci. 8 : 114-127.
Marmigère F, Montelius A, Wegner M, Groner Y, Reichardt LF, Ernfors P (2006). The Runx1/AML1 transcription factor selectively regulates development and survival of TrkA nociceptive sensory neurons. Nat. Neurosci. 9: 180-187.
Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P (2004). Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat. Neurosci. 7: 930-938.
Marti E, Bumcrot DA, Takada R, McMahon AP (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature 375: 322-325.
Marti E, Gibson SJ, Polak JM, Facer P, Springall DR, Van Aswegen G, Aitchison M, Koltzenburg M (1987). Ontogeny of peptide- and amine-containing neurones in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia, and rat skin. J. Comp. Neurol. 266: 332-359.
Martinsen BJ, Bronner-Fraser M (1998). Neural crest specification regulated by the helix-loop-helix repressor Id2. Science 281: 988-991.
Masuda T, Okado N, Shiga T (2000). The involvement of axonin-1/SC2 in mediating notochord-derived chemorepulsive activities for dorsal root ganglion neurites. Dev. Biol. 224: 112-121.
Masuda T, Tsuji H, Taniguchi M, Yagi T, Tessier-Lavigne M, Fujisawa H, Okado N, Shiga T (2003). Differential non-target-derived repulsive signals play a critical role in shaping initial axonal growth of dorsal root ganglion neurons. Dev. Biol. 254: 289-302.
Mattise M (2002). A dorsal elaboration in the spinal cord. Neuron 34: 491-493.
McCarthy PW, Lawson SN (1990). Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene related peptide-like immunoreactivity. Neuroscience 34: 623-632.
McKemy DD, Neuhausser WM, Julius D (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416: 52-58.
McMahon SB, Bennet DLH, Bevan S (2006). Inflammatory mediators and modulators. In: Koltzenburg M, McMahon SB (eds) Wall and Melzack's Textbook of Pain. Elsevier, Edinburgh, pp 49–72.
McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. 12: 1438-1452.
Melzack R, Wall PD (1965). Pain mechanisms: a new theory. Science 150: 971-9.
Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14: 949-959.
Michael G, Averill S, Nitkunan A, Rattray M, Bennett DLH, Yan Q, Priestley JV (1997). Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in trkA-expressing dorsal root ganglion cells and their central terminations within the spinal cord. J. Neurosci. 17: 8476-8490.
Millan MJ (2002). Descending control of pain. Prog. Neurobiol. 66: 355-474.
Miranda-Contreras L, Benítez-Díaz P, Peña-Contreras Z, Mendoza-Briceño RV, Palacios-Prü E (2002). Levels of amino acid neurotransmitters during neurogenesis and in histotypic cultures of mouse spinal cord. Dev Neurosci. 24: 59-70.
Mirnics K, Koerber HR (1995). Prenatal development of rat primary afferent fibers: II. Central projections. J. Comp. Neurol. 355: 601-614.
Molliver DC, Snider WD (1997a). Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. J. Comp. Neurol. 381: 428-438.
Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997b). IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19: 849-861.
Montelius A, Marmigère F, Baudet C, Aquino JB, Enerbäck S, Ernfors P (2007). Emergence of the sensory nervous system as defined by Foxs1 expression. Differentiation 75: 404-417.
Moody WJ (1998). Control of spontaneous activity during development. J. Neurobiol. 37: 97-109.
Moody WJ, Bosma MM (2005). Ion channel development, spontaneous activity, and activity-dependent development in nerve and muscle cells. Physiol. Rev. 85: 883-941.
Müller T, Anlag K, Wildner H, Britsch S, Treier M, Birchmeier C (2005). The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. Genes Dev. 19: 733-743.
Müller T, Brohmann H, Pierani A, Heppenstall PA, Lewis GR, Jessell TM, Birchmeier C (2002). The homedomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34: 551-562.

Munoz-Sanjuan, I and Brivanlou, AH (2002). Neural induction, the default model and embryonic stem cells. Nat. Rev. Neurosci. 3: 271-280.
NagyJI, Hunt SP (1982). Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience 7: 89-97.
Nakamoto K, Shiga T (1998). Tissues exhibiting inhibitory [correction of inhibiory] and repulsive activities during the initial stages of neurite outgrowth from the dorsal root ganglion in the chick embryo. Dev. Biol. 202: 304-314.
Nakamura S, Senzaki K, Yoshikawa M, Nishimura M, Inoue K, Ito Y, Ozaki S, Shiga T (2008). Dynamic regulation of the expression of neurotrophin receptors by Runx3. Development 135: 1703-1711.
Novitch BG, Wichterle H, Jessell TM, Sockanathan S (2003). A requirement for retinoic acid-mediated transcription activation in ventral neural patterning and motor neuron specification. Neuron 40: 81-95.
Olave MJ, Maxwell DJ (2003). Neurokinin-1 projection cells in the rat dorsal horn receive synaptic contacts from axons that possess alpha2C-adrenergic receptors. J. Neurosci. 23: 6837-6846.
Orozco OE, Walus L, Sah DWY, Pepinsky RB, Sanicola M (2001). GFRalpha3 is expressed predominantly in nociceptive sensory neurons. Eur. J. Neurosci. 13: 2177-2182.
Ossipov MH, Dussor GO, Porreca F (2010). Central modulation of pain. J. Clin. Invest. 120: 3779-3787.
Ozaki S, Snider WD (1997). Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J. Comp. Neurol. 380: 215-229.
Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002a). A TRP channel that senses cold stimuli and menthol. Cell 108: 705-715.
Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P, Bevan S, Patapoutian A (2002b). A heat-sensitive TRP channel expressed in keratinocytes. Science 296: 2046-2049.
Perez SE, Rebelo S, Anderson DJ (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. Development 126: 1715-1728.
Pierani A, Brenner-Morton S, Chiang C, Jessell TM (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97: 903-915.
Pierani A, Moran-Rivard L, Sunshine MJ, Littman DR, Goulding M, Jessell TM (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron 29: 367-384.
Pillai A, Mansouri A, Behringer R, Westphal H, Goulding M (2007). Lhx1 and Lhx5 maintain the inhibitory-neurotransmitter status of interneurons in the dorsal spinal cord. Development 134: 357-366.
Pituello F (1997). Neuronal specification: generating diversity in the spinal cord. Curr. Biol. 7: R701-704.
Placzek M (1995). The role of the notochord and floor plate in inductive interactions. Curr. Opin. Genet. Dev. 5: 499-506.
Polgár E, Furuta T, Kaneko T, Todd A (2006). Characterization of neurons that Express preprotachykinin B in the dorsal horn of the rat spinal cord. Neuroscience 139: 687-697.
Porreca F, Ossipov MH, Gebhart GF (2002). Chronic pain and medullary descending facilitation. Trends Neurosci. 25: 319-325.
Portenoy RK, Kanner RM (1996). Definition and assessment of pain. In: Pain management: theory and practice. Edited by Portenoy RK and Kanner RM, Philadelphia, FA Davis Company, pp. 3-18.
Price MP, McIlwrath SL, Xie J, Cheng C, Qiao J, Tarr DE, Sluka KA, Brennan TJ, Lewin GR, Welsh MJ (2001). The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron 32: 1071-1083.
Püschel AW, Adams RH, Betz H (1996). The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. Mol. Cell Neurosci. 7: 419-431.
Qian Y, Shirasawa S, Chen CL, Cheng L, Ma Q (2002). Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1. Genes Dev. 16: 1220-1233.
Rifkin JT, Todd VJ, Anderson LW, Lefcort F (2000). Dynamic expression of neurotrophin receptors during sensory neuron genesis and differentiation. Dev. Biol. 227: 465-480.
Roelink H, Porter JA, Chiang C, Tanabe Y, Chang DT, Beachy PA, Jessell, TM (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. Cell 81: 445-455.
Rubenstein JL, Shimamura K, Martinez S, Puelles L (1998). Regionalization of the prosencephalic neural plate. Annu. Rev. Neurosci. 21: 445-477.
Saito T, Greenwood A, Sun Q, Anderson DJ (1995). Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol. Cell. Neurosci. 6: 280–292.
Schafers M, Lee DH, Brors D, Yaksh TL, Sorkin LS (2003). Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation, J. Neurosci. 23: 3028–3038.
Schoenwolf GC, Smith JL (1990). Mechanisms of neurulation: traditional viewpoint and recent advances. Development 109: 243-270.
Schumacher MA, Jong BE, Frey SL, Sudanagunta SP, Capra NF, Levine JD (2000). The stretch-inactivated channel, a vanilloid receptor variant, is expressed in small-diameter sensory neurons in the rat. Neurosci. Lett. 287: 215-218.
Serbedzija GN, Fraser SE, Bronner-Fraser M (1990). Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. Development 108: 605-612.
Sherrington DS (1906). The integrative action of the nervous system, 2ª Ed, Yale Univ. Press, New Haven.
Sibilla S, Ballerini L (2009). GABAergic and glycinergic interneuron expression during spinal cord development: dynamic interplay between inhibition and excitation in the control of ventral network outputs. Prog. Neurobiol. 89: 46-60.
Silos-Santiago I, Molliver DC, Ozaki S, Smeyne RJ, Fagan AM, Barbacid M, Snider WD (1995). Non-TrkA-expressing small DRG neurons are lost in TrkA deficient mice. J. Neurosci. 15: 5929-5942.
Silvermann JD, Kruger L (1990). Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. J. Neurocytol. 19: 789-801.
Shatz CJ (1983). The prenatal development of the cat's retinogeniculate pathway. J. Neurosci. 3: 482-499.
Shepherd IT, Luo Y, Lefcort F, Reichardt LF, Raper JA (1997). A sensory axon repellent secreted from ventral spinal cord explants is neutralized by antibodies raised against collapsing-1. Development 124: 1377-1385.
Shirasaki R, Pfaff SL (2002). Transcriptional codes and the control of neuronal identity. Annu. Rev. Neurosci. 25: 251-281.
Shubayev VI, Myers RR (2002). Endoneurial remodeling by TNFalph- and TNFalpha-releasing proteases. A spatial and temporal colocalization study in painful neuropathy, J. Peripher. Nerv. Syst. 7: 28–36.
Smith CL (1983). The development and postnatal organization of primary afferent projections to the rat thoracic spinal cord. J. Comp. Neurol. 220: 29-43.
Smith CL, Frank E (1988). Specificity of sensory projections to the spinal cord during development in bullfrogs. J. Comp. Neurol. 269: 96-108.
Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L, Egerton J, Charles KJ, Smart

D, Randall AD, Anand P, Davis JB (2002). TRPV3 is a temperature-sensitive vanilloid receptor-like protein. Nature 418: 186-190.
Spitzer NC (2006). Electrical activity in early neuronal development. Nature 444:707-712.
Stern, CD (2001). Initial patterning of the central nervous system: how many organizers? Nat. Rev. Neurosci. 2: 92-98.
Stewart W, Maxwell DJ (2000). Morphological evidence for selective modulation by serotonin of a subpopulation of dorsal horn cells which possess the neurokinin-1 receptor. Eur. J. Neurosci. 12(12): 4583-4588.
Stone LS, Broberger C, Vulchanova L, Wilcox GL, Hökfelt T, Riedl MS, Elde R (1998).
Differential distribution of alpha2A and alpha2C adrenergic receptor immunoreactivity in the rat spinal cord. J. Neurosci. 18: 5928-5937.
Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112: 819-829.
Streit WJ, Schulte BA, Balentine JD, Spicer SS (1986). Evidence for glycoconjugate in nociceptive primary sensory neurons and its origin from the Golgi Complex. Brain Res. 377: 1-17.
Suzuki R, Morcuende S, Webber M, Hunt SP, Dickenson AH (2002). Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. Nat. Neurosci. 5: 1319-1326.
Tabata T, Takei Y (2004). Morphogens, their identification and regulation. Development 131: 703-712.
Takagi S, Kasuya Y, Shimizu M, Matsuura T, Tsuboi M, Kawakami A, Fujisawa H (1995). Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. Dev. Biol. 170: 207-222.
Tanabe Y, Jessell TM (1996). Diversity and pattern in the developing spinal cord. Science 274: 1115-1123.
Teillet MA, Kalcheim C, Le Douarin NM (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. Dev. Biol. 120: 329-347.
Timmer, JR, Wang, C and Niswander, L (2002). BMP signalling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. Development 129: 2459-2472.
Tracey I, Manthy PW (2007). The cerebral signature for pain perception and its modulation Neuron 55: 377-391.
Treede RD, Meyer RA, Raja SN, Campbell JN (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. Prog Neurobiol. 38: 397-421.
Todd AJ. McKenzie J (1989). GABA-imunoreactive neurons in the dorsal horn of the rat spinal cord. Neuroscience 31: 799-806.
Todd AJ, Puskar Z, Spike RC, Hughes C, Watt C, Forrest L (2002). Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. J. Neurosci. 22: 4103-4113.
Todd AJ, Spike RC (1993). The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog. Neurobiol. 41: 609-645.
Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21: 531-543.
Ulloa F, Brisco J (2007). Morphogens and the control of cell proliferation and patterning in the spinal cord. Cell Cycle 6: 2640-2649.
van Praag H, Frenk H (1991). The development of stimulation-produced analgesia (SPA) in the rat. Brain Res. Dev. Brain Res. 64: 71-76.
Vriens J, Appendino G, Nilius B (2009). Pharmacology of vanilloid transient receptor potential cation channels. Mol. Pharmacol. 75: 1262-1279.
Waldmann R, Bassilana F, de Weille J, Champigny G, Heurteaux C, Lazdunski M (1997). Molecular cloning of a non-inactivating proton-gated Na+ channel specific for sensory neurons. J. Biol. Chem. 272: 20975-20978.
Watkins LR, Milligan ED, Maier SF (2001). Glial activation: a driving force for pathological pain. Trends Neurosci. 24: 450–455.
Watkins LR, Milligan ED, Maier SF (2001). Spinal cord glia: new players in pain. Pain 93: 201–205.
Weber ED, Stelzner DJ (1977). Behavioral effects of spinal cord transection in the developing rat. Brain Res 125: 241-255.
Wei F, Dubner R, Zou S, Ren K, Bai G, Wei D, Guo W (2010). Molecular depletion of descending serotonin unmasks its novel facilitatory role in the development of persisten pain. J. Neurosci. 10: 8624-8636.
White FA, Behar O (2000). The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. Dev. Biol. 225: 79-86.
Wichterle H, Lieberam I, Porter JA, Jessell TM (2002). Directed differentiation of embryonic stem cells into motor neurons. Cell 110: 385-397.
Wilson SI, Edlund T (2001). Neural induction: towards a unifying mechanism. Nat. Neurosci. Suppl. 4: 1161-1168.
Wilson SW, Houart C (2004). Early steps in the development of the forebrain. Dev. Cell 6: 167-181.
Wilson L, Maden M (2005). The mechanisms of dorsalventral patterning in the vertebrate neural tube. Dev. Biol. 282: 1-13.
Wine-Lee L, Ahn KJ, Richardson RD, Mishina Y, Lyons KM, Crenshaw III EB (2004). Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. Development 131: 5393-5403.
Wirkner K, Sperlagh B, Illes P (2007). P2X3 receptor involvement in pain states.
Mol. Neurobiol. 36: 165-183.
Woolf CJ, Salter MW (2000). Neuronal plasticity: increasing the gain in pain. Science 88: 1765–1768.
Wu WL, Ziskind-Conhaim L, Sweet MA (1992). Early development of glycine- and GABA-mediated synapses in rat spinal cord. J Neurosci. 12: 3935-3945.
Xu H, Ramsey IS, Kotecha SA, Moran MM, Chong JA, Lawson D, Ge P, Lilly J, Silos-Santiago I, Xie Y, DiStefano PS, Curtis R, Clapham DE (2002). TRPV3 is a calcium-permeable temperature-sensitive cation channel. Nature 418: 181-186.
Xu Y, Lopes C, Qian Y, Liu Y, Cheng L, Goulding M, Turner EE, Lima D, Ma Q (2008). Tlx1 and Tlx3 coordinate specification of dorsal horn pain-modulatory peptidergic neurons. J Neurosci. 28: 4037-4046.
Yoshikawa M, Senzaki K, Yokomizo T, Takahashi S, Ozaki S, Shiga T (2007). Runx1 selectively regulates cell fate specification and axonal projections of dorsal root ganglion neurons. Dev. Biol. 303:663-674.
Zhou Q, Anderson DJ (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109:61-73.
Zhuo M, Gebhart GF (1991). Spinal serotonin receptors mediate descending facilitation of a nociceptive reflex from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. Brain Res. 550: 35-48.
Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16: 109-110.
Zuellig RA, Rader C, Schroeder A, Kalousek MB, Von Bohlen und Halbach F, Osterwalder T, Inan C, Stoeckli ET, Affolter HU, Fritz A, Hafen E, Sonderegger P (1992). The axonally secreted cell adhesion molecule, axonin-1. Primary structure, immunoglobulin-like and fibronectin-type-III-like domains and glycosyl-phosphatidylinositol anchorage. Eur. J. Biochem. 204: 452-463.
Zwick M, Davis BM, Woodbury J, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002). Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J. Neurosci. 22: 4057-4065.

II

PUBLICATIONS

DRG11 Immunohistochemical Expression During Embryonic Development in the Mouse

Sandra Rebelo, Carlos Reguenga, Liliana Osório, Carlos Pereira, Claúdia Lopes, and Deolinda Lima*

DRG11 is a paired domain transcription factor that is necessary for the assembly of the nociceptive circuitry in the spinal cord dorsal horn. It is expressed in small dorsal root ganglion (DRG) neurons and in their projection area in the spinal cord. *Drg11* knockout mice exhibit structural and neurochemical defects both at the DRG and spinal superficial dorsal horn and present reduced nociceptive responses. In this study, a polyclonal antibody against DRG11 was generated and used for a detailed systematic spatio-temporal analysis of DRG11 expression during development. DRG11 is first detected at E10.5 in the spinal dorsal horn, DRG and trigeminal ganglion, where it persists until P14-21. At the cranial level, DRG11 expression is observed from E10.5 up to the same early post-natal ages in several cranial sensory ganglia and brain nuclei. These results suggest that DRG11 is required for the establishment of the first neuronal sensory relay along development. *Developmental Dynamics 236:2653–2660, 2007.* © 2007 Wiley-Liss, Inc.

Key words: development; DRG11; mouse; spinal cord; Prrxl1; brainstem; homeodomain

Accepted 20 June 2007

INTRODUCTION

Spinal cord dorsal horn neurons are responsible for modulating and relaying various types of sensory information to higher brain centers using a complex, still poorly understood circuitry that is established during embryonic development (for review, see Gillespie and Walker, 2001; Julius and Basbaum, 2001; Goulding et al., 2002). In the brain, somatic sensory information is relayed through the trigeminal nuclei, while visceral sensory information is relayed through the solitary nucleus (Saper, 2000). The mechanisms of differentiation of the various sensory relay neurons in the hindbrain and spinal cord began to be understood only recently mainly due to the lack of specific molecular markers. At the spinal cord level, DRG11, a paired-like homeodomain protein, is detected in primary sensory neurons and in their projection area in the superficial dorsal horn (Saito et al., 1995). Because superficial dorsal horn laminae are involved in nociception, *Drg11* was proposed to be required for the development of the nociceptive system. This hypothesis was later supported by the finding that *Drg11* knockout mice (*Drg11*$^{-/-}$) exhibit reduced reflex responses to painful stimuli together with abnormalities in superficial dorsal horn structure and neurochemistry (Chen et al., 2001). Failure to establish normal vibrisal somatosensory maps in the thalamus and SI cortex was also demonstrated (Ding et al., 2003). A recent study addressing the expression of various primary afferent neuronal markers along development pointed to a role for DRG11 in early postnatal survival of apparently normally differentiated small primary afferent neurons innervating various kinds of peripheral tissues (Rebelo et al., 2006).

In the present study, a polyclonal antibody was generated against the C-terminal part of mouse DRG11 and used to carry out a detailed systematic spatio-temporal analysis of DRG11 expression along embryonic development. The results indicate that DRG11 is expressed early in the embryo both in central ner-

Laboratory of Molecular Cell Biology, Faculty of Medicine of Oporto and IBMC-Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal
Grant sponsor: FCT; Grant number: POCTI/1999/NSE/32357; Grant number: POCI/SAU-NEU/59093/2004.
***Correspondence to: Deolinda Lima, Instituto de Histologia e Embriologia, Faculdade de Medicina do Porto, Alameda Hernani Monteiro, 4200-319 Porto, Portugal. E-mail: limad@med.up.pt**

DOI 10.1002/dvdy.21271
Published online 5 August 2007 in Wiley InterScience (www.interscience.wiley.com).

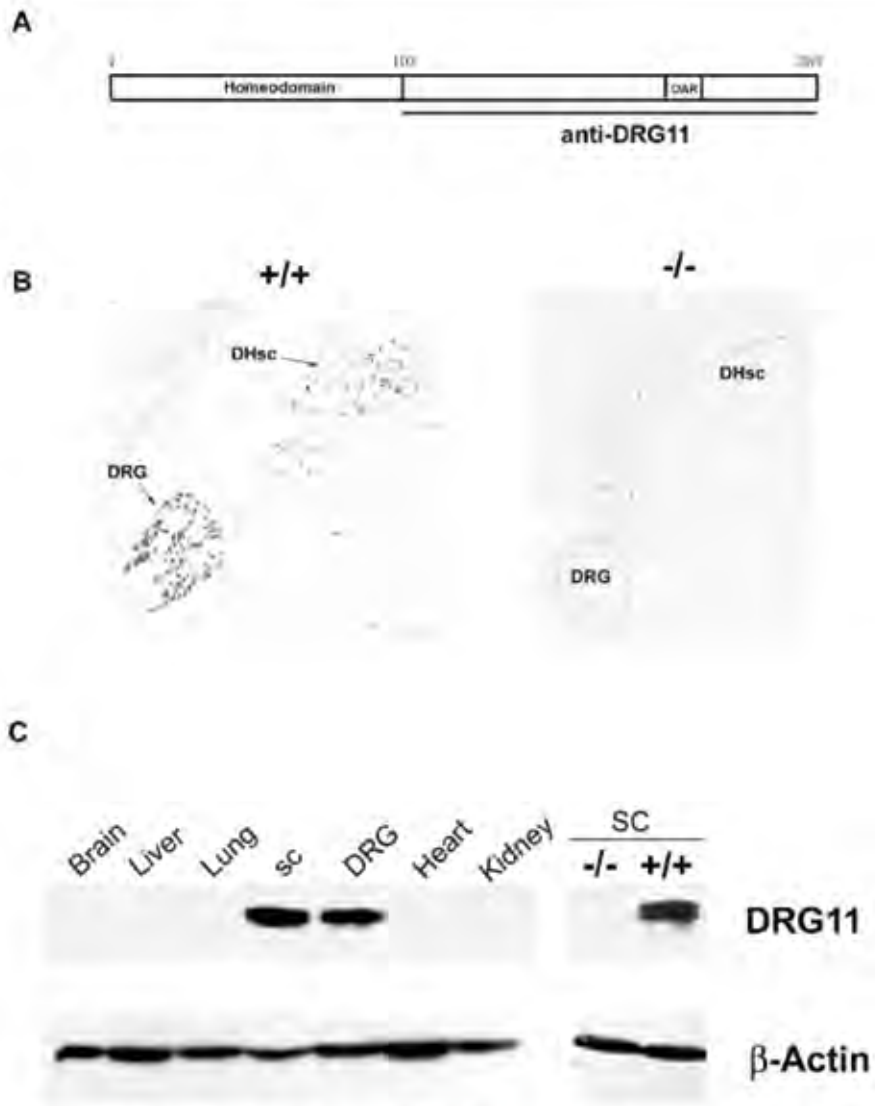

Fig. 1. Characterization of the antibody raised against DRG11, a paired-like homeodomain protein. **A:** Schematic representation of *Drg11* structure, which includes a DNA-binding homeodomain and an OAR domain. The region of amino acids 103–263 was used for antibody generation. **B:** Expression of DRG11 protein in wild-type (+/+) and *Drg11*$^{-/-}$ mice (−/−) at embryonic day (E) 18.5. Arrows indicate expression of DRG11 in the dorsal horn of the spinal cord (DHsc) and the dorsal root ganglia (DRG). **C:** Western blotting analysis of DRG11 expression pattern. Several tissue extracts were immunoblotted for DRG11, but bands were obtained only from the DRG and spinal cord (sc) of wild-type mice. Beta-actin was blotted on the same membrane and used as a sample loading control.

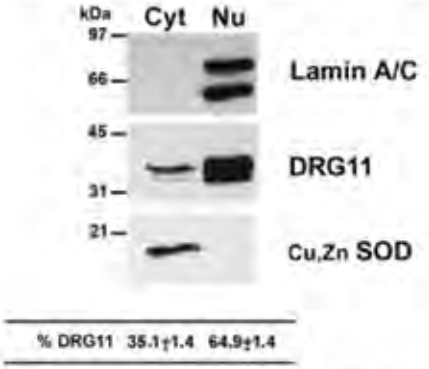

Fig. 2. Subcellular expression of DRG11, a paired-like homeodomain protein. Cells from spinal cords of embryonic day (E) 18.5 wild-type mice were fractionated by differential centrifugation into cytoplasmic (cyt) and nuclear (nu) fractions. Fifty micrograms of each fraction was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the presence of DRG11 was detected by immunoblotting. Lamin A/C and Cu, Zn SOD were used as, respectively, nucleus and cytoplasmic marker proteins to validate the fractionation. Values represent the mean ± SEM of three replicate experiments.

vous system (CNS) and peripheral nervous system (PNS) neurons subserving somatosensory and viscerosensory functions. The protein is first immunodetected at embryonic day 10.5 (E10.5) and is maintained throughout development. It is expressed in the dorsal root ganglia (DRG), trigeminal, facial, vestibulocochlear, glossopharyngeal, and vagus cranial ganglia, spinal superficial dorsal horn and sensory hindbrain nuclei. Postnatally, DRG11 expression progressively decreases, being undetectable by P21. These findings suggest that DRG11 plays a crucial role in the development of primary afferent neurons and second-order sensory neurons.

RESULTS AND DISCUSSION

Generation and Characterization of the Antibody Directed Against DRG11

Drg11 expression has first been detected by in situ hybridization in the DRG and the spinal cord dorsal horn (Saito et al., 1995; Chen et al., 2001). To gain further insight on DRG11 function, we generated a rabbit anti-DRG11 antibody and tested its specificity by immunostaining the DRG and spinal cord of wild-type and *Drg11*$^{-/-}$ E18.5 embryos. The antibody was raised against the C-terminal region, spanning amino acids 103 to 263, which excludes the homeodomain (Fig. 1A). Immunohistochemical analysis showed that the protein expression pattern correlates entirely with the previously reported mRNA expression in the nervous system (Saito et al., 1995; Chen et al., 2001), and no expression was detected in any other tissues (Fig. 1B). DRG11 immunostaining was observed in the DRG and in the spinal cord superficial dorsal horn of wild-type animals, but not in the *Drg11*$^{-/-}$ mice (Fig. 1B). Moreover, immunoblotting analysis of protein extracts from several tissues of wild-type mice revealed that the expression of DRG11 is present in DRG and spinal cord (Fig. 1C). As expected, no signal was observed in the spinal cord of *Drg11*$^{-/-}$ mice (Fig. 1C). Although DRG11 has a predicted molecular mass of 28 kDa, it was detected by immunoblotting around 36 kDa (Fig. 2). Such an altered migration in

TABLE 1. Expression Analysis of DRG11 in the Peripheral and Central Mouse Nervous System at Various Time Points Along Embryonic Development and Postnatal Life[a]

	Developmental age							
Nervous system	10.5	12.5	15.5	18.5	P0	P7	P14	P21
Peripheral								
Dorsal root ganglia	+	+	+	+	+	+	(+)	–
Trigeminal (V) ganglia	+	+	+	+	+	+	(+)	–
Facial (VII) ganglia	+	+	+	+	+	na	na	na
Vestibulocochlear (VIII) ganglia	+	+	+	+	+	na	na	na
Glossopharyngeal (IX) ganglia	+	+	+	+	+	na	na	na
Vagus (X)	+	+	+	+	+	na	na	na
Central								
Spinal cord	+	+	+	+	+	+	(+)	–
Spinal trigeminal nucleus	–	+	+	+	+	+	(+)	–
Principal trigeminal nucleus	–	+	+	+	+	+	(+)	–
Nucleus tractus solitarus	–	+	+	+	+	+	(+)	–
Prepositus nucleus	–	+	+	+	+	+	(+)	–

[a]Indicated are the relative amounts of DRG11, a paired-like homeodomain protein, present in each region. Symbols: +, strong expression; (+), weak expression; –, no expression; na, not analyzed.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is occasionally observed in homeodomain proteins (Jackson et al., 2002). Nonetheless, the signal must correspond to DRG11, because it is located in the same areas as *Drg11* mRNA and in *Drg11*$^{-/-}$ littermates no immunostaining or immunoblotting bands were detected. All together, these findings indicate that the antibody specifically recognizes the DRG11 protein.

To further characterize the anti-DRG11 antibody, we addressed the subcellular localization of DRG11 in the spinal cord of embryonic day (E) 18.5 wild-type embryos. Immunoblotting revealed a pronounced DRG11 signal in the nucleus and weaker staining in the cytosol. Nuclear and cytosolic fractions were isolated to quantitatively address this difference. Each fraction was resolved in SDS-PAGE and immunoblotted with the anti-DRG11 antibody. DRG11 expression amounted to approximately two-thirds (64.9%) in the nucleus and one-third (35.1%) in the cytosol (Fig. 2).

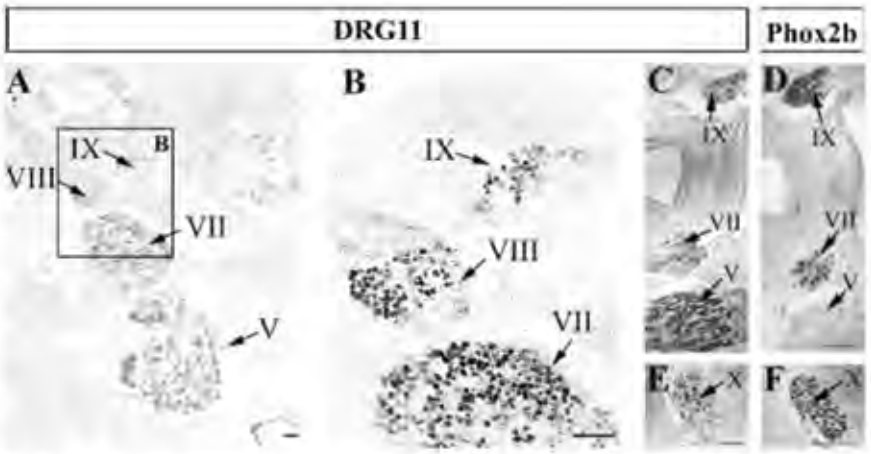

Fig. 3. Expression of DRG11, a paired-like homeodomain protein, in the mouse cranial ganglia. **A:** Trigeminal (V), facial (VII), vestibulocochlear (VIII), and glossopharygeal (IX) ganglia at embryonic day (E) 18.5. **B:** Higher magnification of the boxed region in A. **C–F:** Sagittal adjacent sections of E11.5 facial (C,D), glossopharygeal (C,D), and vagus (X; E,F) visceral sensory ganglia showing DRG11 expression (C,E) and Phox2b expression (D,F). Scale bars = 100 μm.

DRG11 Expression Pattern During Nervous System Development

Previous in situ hybridization data from mice (Saito et al., 1995; Chen et al., 2001; Qian et al., 2002; Ding et al., 2003) pointed to the restricted expression of *Drg11* in the DRG, spinal cord dorsal horn, and trigeminal brainstem complex. However, to date, no comprehensive overview of DRG11 expression in the entire embryo and how it evolves along the development of the nervous system was attained. We sought to carefully analyze the DRG11 expression in the entire neuroaxis at various time points of embryonic development and early postnatal life by taking advantage of our novel generated antibody. The study was carried out in mice from the time of neural tube closure (E10.5) up to adulthood. DRG11 immunostaining was observed in the DRG, spinal cord, cranial sensory ganglia, and brainstem sensory nuclei (results are summarized in Table 1).

DRG11 Expression in Sensory Ganglia

DRG11 was immunodetected in the DRG and the trigeminal (V) ganglia (both the ophthalmic and maxilloman-

dibular lobes) throughout development, beginning at age E10.5 (Table 1; Fig. 3A,C). DRG11 expression was also observed in other cranial sensory ganglia, namely the facial (VII), vestibulocochlear (VIII), glossopharyngeal (IX), and vagus (X; Table 1; Fig. 3A–C,E). Immunostaining persisted in all areas with no apparent changes until perinatal age (P0). Postnatally, due to difficulties in dissecting the cranial ganglia, DRG11 expression was only sought in the DRG and trigeminal ganglia. In both structures, DRG11 expression was still present, although it was lower at P14 and nil at P21 (Table 1).

DRG11 expression in the DRG and the trigeminal ganglia had been previously reported (Ding et al., 2003; Rebelo et al., 2006). The trigeminal ganglion is analogous to the DRG, containing the cell bodies of somatic sensory fibers from the head and oral cavity. As in the DRG, trigeminal primary sensory neurons are distributed through large size and small size populations. DRG11 was exclusively expressed in small neurons in both the trigeminal ganglion and the DRG. Coimmunostaining for DRG11 with either trkA, CGRP, or IB4 showed colocalization with the three markers of small DRG neurons, which supports a role for these neurons in nociceptive and thermal sensations (Fig. 4A–C).

The facial, glossopharyngeal, and vagus ganglia contain visceral primary afferent neurons and convey nociceptive input to the spinal trigeminal nucleus. To confirm that DRG11 is expressed in visceral ganglia, we immunostained adjacent sections with Phox2b, a molecular visceral sensory marker (Fig. 3D–F). DRG11 expression was present in all Phox2b-positive visceral ganglia. Because the available DRG11 and Phox2b antibodies are raised in the same species, colocalization of the two markers at the cellular level was not addressed. In agreement with what was reported in the zebrafish (McCormick et al., 2007), expression of DRG11 seemed to be weaker in these visceral sensory ganglia compared with the somatic trigeminal ganglion. No DRG11 expression was detected in cranial ganglia exclusively subserving motor function.

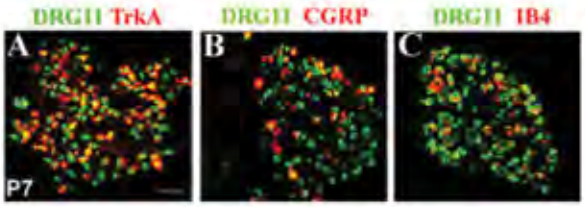

Fig. 4. Expression of DRG11, a paired-like homeodomain protein, in the mouse dorsal root ganglion. **A–C:** DRG11 coexpresses with trkA (A) and CGRP (B) in small peptidergic DRG neurons and with IB4 (C) in nonpeptidergic neurons. Scale bars = 50 μm.

DRG11 Expression in the Brain

In the brain, expression of DRG11 was first observed in the principal trigeminal nucleus (Pr5) at E12.5 (Table 1; Fig. 5A,B,G,H), followed by the spinal trigeminal nucleus (subnucleus caudalis and subnucleus oralis; Table 1; Fig. 5C,G,H). DRG11 was also detected in the nucleus of the solitary tract (NTS) and nucleus prepositus (NP; Table 1; Fig. 5D–F,I). From P7 on, DRG11 expression decreased, being virtually absent by age P21 (Table 1).

All the brainstem nuclei expressing DRG11 function as first-relay stations

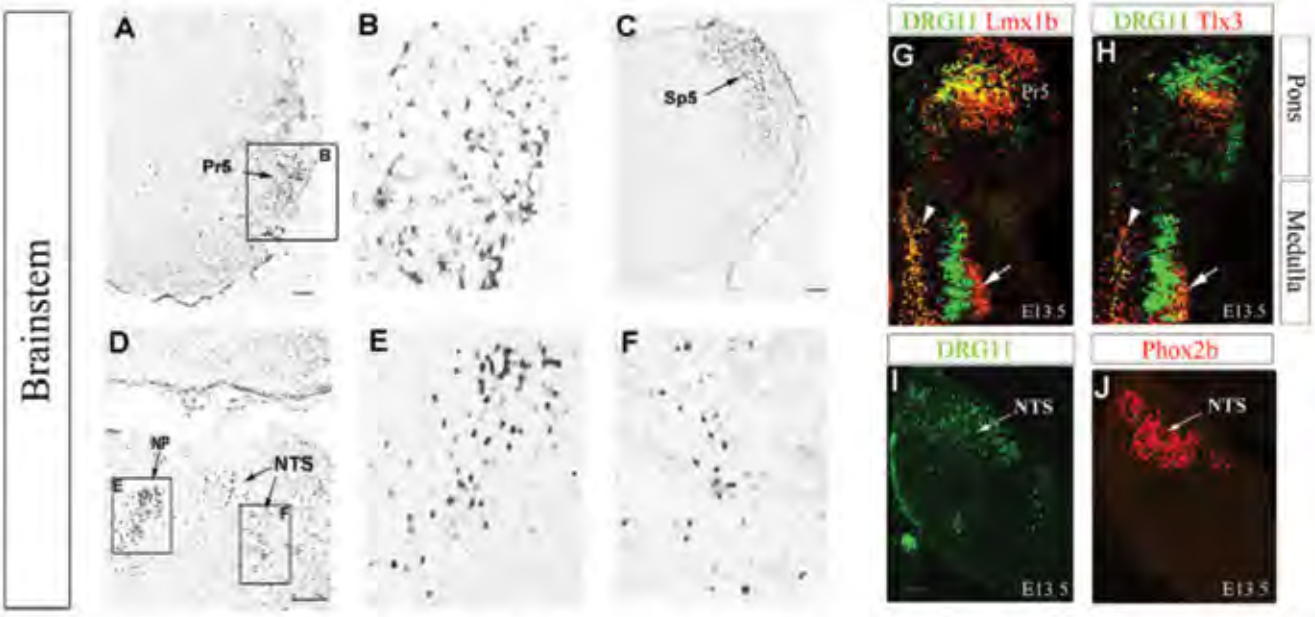

Fig. 5. Expression of DRG11, a paired-like homeodomain protein, in the mouse brain. **A,B:** Principal trigeminal nucleus (Pr5). B: Magnification of the boxed region in A. **C:** Spinal trigeminal nucleus (Sp5). **D–F:** Nucleus of the solitary tract (NTS; D,F) and nucleus prepositus (NP; D,E). A–F: Taken from embryonic day (E) 18.5 embryos. **G,H:** Expression of DRG11and Lmx1b (G) and Drg11 and Tlx3 (H) in the prospective Pr5 (pons) and medulla at E13.5 (horizontal sections). **I,J:** Expression of DRG11 (I) and Phox2b (J) on adjacent sections at E13.5 (coronal sections). Scale bars = 100 μm in A–E; 50 μm in G–J.

for sensory input arriving through cranial nerves. The trigeminal system is made up of various components processing different kinds of somatic peripheral input. The subnucleus caudalis of the spinal trigeminal nucleus processes mainly nociceptive and thermal information, and the principal trigeminal nucleus processes tactile information. Among trigeminal areas that did not express DRG11 is the subnucleus interpolaris, which is less well characterized but thought to be involved in tactile-evoked reflex activity.

Expression of DRG11 in the spinal trigeminal nucleus (Sp5) is in accordance with previous findings by Ding et al. (2003) and supports the assumption that DRG11 plays an important role in the development of the nociceptive neuronal circuit at its first relay (Chen et al., 2001; Rebelo et al., 2006). However, DRG11 is also expressed very early in a trigeminal area devoted to tactile sensation, the principal nucleus (Qian et al., 2002; Ding et al., 2003; present results), raising the possibility that DRG11 may be also required for the establishment of the sensory innocuous processing circuit. Unfortunately, the capability of experimentally testing tactile functioning is very limited.

Immunohistochemical colocalization of DRG11 with either Lmx1b or Tlx3, markers expressed in both trigeminal nuclei (Qian et al., 2002; Ding et al., 2003), was assessed. The majority of DRG11 neurons in the principal nucleus colocalized with Lmx1b (Fig. 5G), while only a small portion colocalized with Tlx3 (Fig. 5H). In the medulla, three immunoreactive columns were depicted. A lateral column, which is likely to originate the Sp5 was densely populated by neurons double-stained for DRG11 and either Lmx1b or Tlx3 (Fig. 5G–H, arrowhead). At an intermediate position laid a second column with neurons that only stained for DRG11 (Fig. 5G,H). More medially neurons also colocalized DRG11 and Lmx1b/Tlx3, although the proportion of double-stained neurons was not as abundant as laterally (Fig. 5G,H, arrow). According to the localization of Lmx1b and Tlx3 at the age of E13.5–E14.5 (Qian et al., 2001; Dauger et al., 2003), the two medial columns may be at the origin of the NTS.

The NTS is targeted by visceral afferent fibers supporting a role for DRG11 in the development of visceral sensory systems. Its formation during development is dependent on the expression of Tlx3, which is upstream of *Drg11* (Qian et al., 2001). DRG11 coimmunostaining with Lmx1b or Tlx3 indicates that, earlier in development, there is a population in the NTS that is DRG11-specific and a another that colocalizes extensively either with Lmx1b or Tlx3 (Fig. 5G,H). Phox2b has been used as a molecular marker for visceral neurons during their development and migration (Dauger et al., 2003). Immunostaining for both transcription factors on adjacent sections showed a partial overlap, indicating that, although most NTS neurons are DRG11-positive, there is a fraction that is not (Fig. 5I,J). As to the nucleus prepositus, it is thought to play a role in the regulation of eye movements. Interestingly, *Drg11*$^{-/-}$ mice exhibit defects in eye movement control shortly after birth. The data here collected on the brain expression of DRG11 clearly indicate that *Drg11* is preferentially involved in the development of sensory processing areas from early differentiation stages until shortly after birth.

DRG11 Expression in the Spinal Cord

In the spinal cord, DRG11 expression was first detected at E10.5 in dI3 and dI5 neurons (Fig. 6A). This stage corresponds to the first wave of neurogenesis, which specifies the dorsal neural tube into six classes of early-born neurons (dI1-6) according to their dorsoventral position and expression profile of some homeodomain proteins (Liem et al., 1997; Lee and Jessell, 1999; Caspary and Anderson, 2003; Helms and Johnson, 2003). Periventricular staining decreased at E11.5 (Fig. 6B) to increase again at E12.5 (Fig. 6C). At this time point, staining was massive of the dorsal periventricular area and extended to the dorsolateral spinal cord region (Fig. 6C). Most of these neurons are likely to represent the late born-populations, which will populate the spinal cord superficial laminae (Gross et al., 2002; Matisse, 2002; Müller et al., 2002). At E11.5, a few neurons could be observed migrating ventrally (Fig. 6B, arrows). Ventrally located neurons were detected until E15.5 (Fig. 6B–F, arrows). Recently, it was suggested that early-born neurons (born between E10.5 and E11.5) migrate ventrally and do not contribute to the formation of the superficial dorsal horn (Caspary and Anderson, 2003). At E13.5–E14.5, DRG11-positive neurons were packed in the lateral most portion of the superficial spinal cord, immunostaining in the periventricular zone being still detected, but much less intensely (Fig. 6D,E). From E15.5 on, DRG11 neurons occupied the most superficial region of the spinal cord (Fig. 6F), so that at E18.5, when spinal cord lamination is distinguishable, DRG11 expression extended from lamina I to III (Fig. 6G). This expression pattern was maintained postnatally (Fig. 6H–K), although decreasing in intensity along time (Fig. 6H,I). Even though Lmx1b and Tlx3 expression spanned the entire dorsal horn of the spinal cord, DRG11 expression was restricted to the superficial spinal cord laminae (Fig. 6L,M). In addition, almost all DRG11-expressing neurons were Lmx1b-positive (Fig. 6L), while colocalization with Tlx3 was observed in a lesser extent (Fig. 6M).

The superficial dorsal horn, in particular laminae I and II, has for long been claimed to process nociceptive information. DRG11-positive spinal neurons heavily populate this spinal area and are, therefore, likely to constitute an important component of the spinal nociceptive circuitry. In this light, it should be recalled that, in *Drg11*$^{-/-}$ mice, small peptidergic and nonpeptidergic primary afferent neurons appear to develop normally until birth but undergo apoptosis immediately after, decreasing their numbers to approximately half their normal amount (Rebelo et al., 2006). Because primary afferent fibers establish connections with superficial spinal neurons at late stages of embryonic development (Reynolds et al., 1991; Fariñas et al., 1996; White et al., 1996), it is possible that this apoptotic fate is due to the absence of their neuronal spinal targets. It remains to be clarified, however, whether ablation of spinal

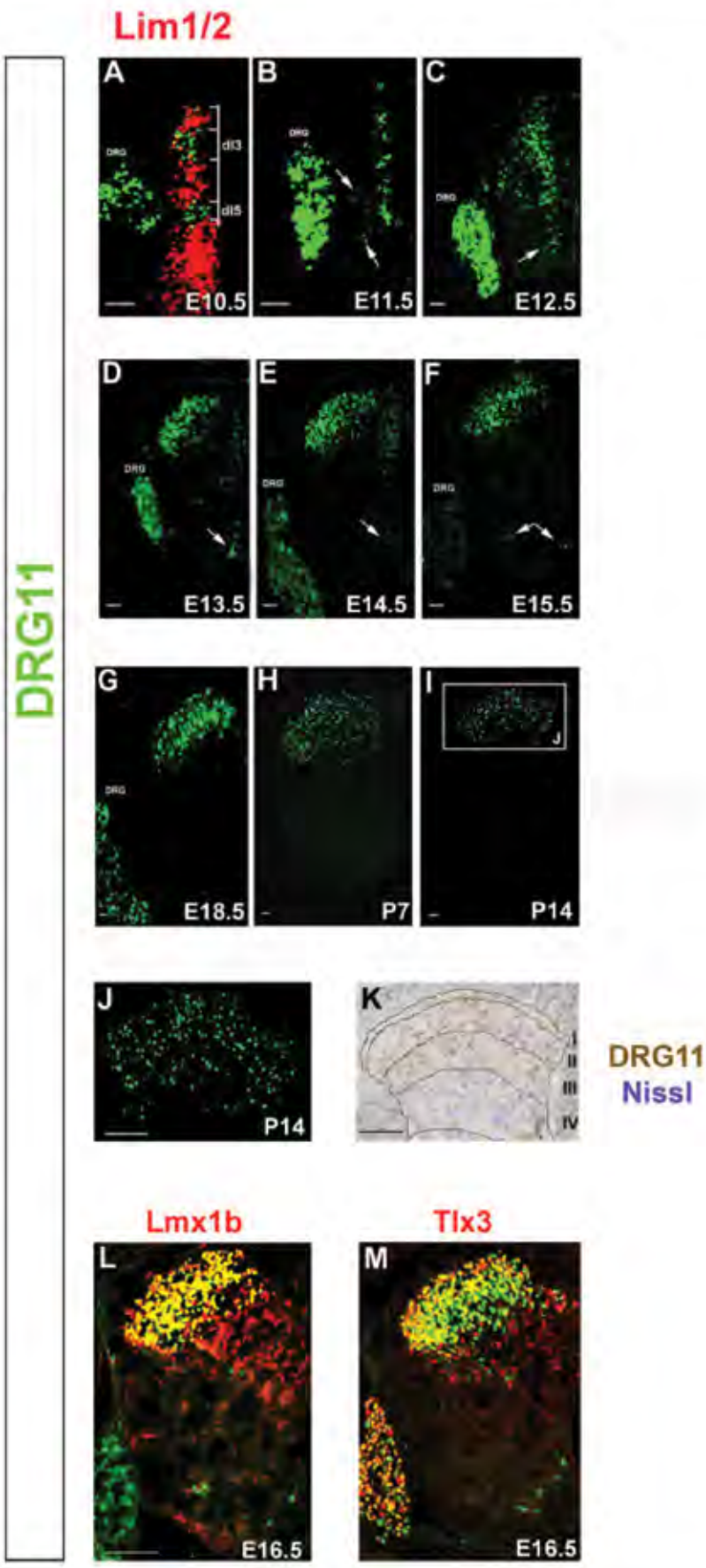

Fig. 6.

second-order neurons is the primary trigger of nociceptive disruption in these mice.

Also relevant is the here found expression of DRG11 in neurons of lamina III. Lamina III is the site of termination of Aδ D-hair follicle primary afferents (Light and Perl, 1979; Willis et al., 2004), which probably suggests that processing of innocuous input may also be, at least partly, dependent on DRG11. In this respect, it is worth mentioning that brain areas devoted to tactile processing, such as the principal trigeminal nucleus, heavily express DRG11 at early stages of embryonic development.

Semiquantitative Characterization of DRG11 Along Development

To access the relative expression of DRG11 along development, a time course immunoblotting analysis was performed using spinal cord protein extracts from mice at E15.5, E18.5, P0, P7, P14, and P21 (Fig. 7). Developmental ages before E15.5 were not considered due to the difficulty in micro-dissecting correctly the neural tube of these embryos. At E15.5 and E18.5, expression of DRG11 was very high. It was reduced to approximately half at birth and kept decreasing until P14, where the levels of DRG11 were almost nil (Fig. 7). In the adult spinal cord (P21), no DRG11 was detected by immunoblotting. The DRG11 signal

Fig. 6. Time course analysis of the expression of DRG11, a paired-like homeodomain protein, in the developing spinal cord. **A:** DRG11 is first expressed in dI3 and dI5 interneurons at embryonic day (E) 10.5. Lim1/2 was used as a dI 2, 4, 6 marker. **B,C:** At E11.5 and E12.5, DRG11 is detected in the periventricular zone and neuronal migration is initiated. **D,E:** DRG11 expression starts to be concentrated in laminae I–III neurons. Periventricular staining is still detected. **F,G:** The migration of DRG11-expressing neurons is settled. **H,I:** Postnatally, DRG11-positive neurons are localized predominantly in laminae I–III, but DRG11 expression progressively decreases. **J:** Higher magnification of the boxed region shown in I. **K:** Laminae I–IV were depicted by Nissl (blue) staining at P14 to show the location at laminae I–III of DRG11-positive neurons (brown). **L:** DRG11-positive neurons are mostly Lmx1b-positive and are restricted to the superficial laminae I–III. **M:** Neurons expressing both Drg11 and Tlx3 are preferentially located in lamina II of the superficial spinal dorsal horn. Scale bars = 50 μm.

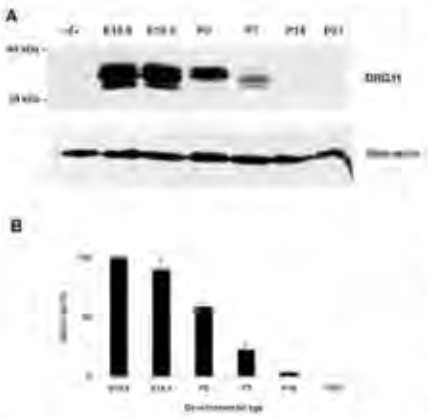

Fig. 7. Western-blotting analysis of DRG11, a paired-like homeodomain protein, expression along development. **A:** Time-course analysis of DRG11 expression was performed using mouse spinal cord extracts from embryonic day (E) 15.5, E18.5 and postnatal ages P0, P7, P14, and P21. Knockout mice were used as negative control. **B:** Relative quantification of DRG11 expression at the various time points was compared taking E15.5 expression as 100%.

was normalized with beta-actin, used as a loading control.

In this study, we used an anti-DRG11 antibody generated in our laboratory to address the immunohistochemical expression pattern of the homeodomain transcription factor DRG11 during development in the mouse. We show that DRG11 expression is restricted to the nervous system and occurs in peripheral ganglia as well as in brain and spinal cord areas related to the processing of somatosensory and viscerosensory information. In the spinal cord, DRG11 expression coincides with the two waves of neurogenesis, defining two subpopulations of early-born (dI3 and dI5) as well as late-born neurons. As development proceeds, DRG11-positive neurons settle in layers I–III of the spinal cord dorsal horn.

EXPERIMENTAL PROCEDURES

Genotyping and Maintenance of Animals

Drg11 mutant mice were generated by intercross between heterozygous mice. Wild-type mice and *Drg11*$^{-/-}$ littermates were genotyped as previously described (Chen et al., 2001). Animals used in this study were bred and maintained at the IBMC animal facility. The day when the vaginal plug was formed was considered to be the E0.5. The ethical guidelines for investigation of experimental pain in animals (Zimmermann, 1983) and the European Community Council Directive of 24 November 1986 (86/609/EEC) were followed.

Generation of Anti-Drg11 Antisera

A 480 bp cDNA fragment from the murine *Drg11*, corresponding to amino acids 103 to 263 (excluding the homeobox domain), was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from total RNA of newborn spinal cord using the one-shot RT-PCR kit (Roche) following the manufacturer's instructions. The following primers were used: 5′-aaggaacccatggcagag-3′ and 5′-tcatacactcttctctccctcgc-3′. The amplified PCR fragment was cloned using the TA cloning kit (Invitrogen) into the pCR2.1 plasmid, and digested with the *Xba*I and *Bam*HI restriction enzymes to subclone the *Drg11* cDNA fragment into the bacterial expression vectors pGEX-4T3 (Amersham Biosciences) and pRSET-A (Invitrogen) in frame with glutathione-*S*-transferase (GST) and (His)6-tag sequences, respectively. The fusion proteins (His)6-*Drg11*(C-terminal)andGST-DRG11(C-terminal) were propagated in BL21-(DE3)pLysS *Escherichia coli* cells and affinity purified on nickel or GST resins (Sigma), respectively, according to the manufacturer's recommendations. The purified (His)6-*Drg11*(C-terminal) recombinant protein was injected into rabbits. The antiserum was affinity purified using a CNBr-activated resin (Amersham Biosciences) conjugated with the GST-DRG11 (C-terminal) recombinant protein according to the manufacturer's protocol.

Tissue Preparation

Embryos were removed by cesarian surgery of pregnant females under anesthesia (sodium pentobarbital 50 mg/kg intraperitoneally), fixed in 4% paraformaldehyde for at least 4 hr, cryoprotected in 30% sucrose overnight, and sectioned on a cryostat at 12 μm. Mice at postnatal ages were also anesthetized before perfusion through the ascending aorta with phosphate-buffered saline 0.1 M (PBS) followed by 4% paraformaldehyde. The spinal cord, DRG, and brain were dissected, post-fixed for 2 hr, cryoprotected in 30% sucrose overnight and embedded in Jung Tissue Freezing Medium before being sectioned into 12-μm sections in a cryostat.

Subcellular Fractionation and Western Blotting

Nuclear and cytosolic fractions were obtained as previously described (Manitt et al., 2001). Briefly, in a typical experiment, 500 μg of spinal cord protein extract were homogenized in SEI buffer containing 10 mM imidazole, pH 7.2, 5 mM ethylenediaminetetraacetic acid, 0.32 M sucrose and protease inhibitor cocktail (Sigma), and centrifuged at 1,000 × *g* for 10 min at 4°C. Nuclear pellet was washed three times in SEI buffer, and the supernatant was centrifuged at 16,000 × *g* for 10 min at 4°C, to obtain the cytosolic fraction. Of the total protein, 51% was recovered in the cytosolic fraction, whereas 26% was found in the nuclear fraction. These values were considered for the estimation of the amount of DRG11 in each fraction.

For Western blotting analysis, protein samples were resolved in a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad). Immunoblot analysis was performed using classic protocols and signal was detected with the Immun-Star Chemiluminescent kit (Bio-Rad). Signal intensities were determined using the 1D Image Analysis Software (Kodak), and normalized with beta-actin. The values were presented as a mean of three independent experiments (n = 3).

Immunohistochemistry

For immunohistochemical staining, tissue endogenous peroxidase was quenched in PBS containing 10% methanol and 3% hydrogen peroxide. Sections were immersed in PBS containing 0.4% Triton X-100 and 10% normal serum from the host species of the secondary antibody to be used (see below), followed by immersion overnight in rabbit anti-DRG11 (1:500) or rabbit anti-Phox2b (gift from Qiufu Ma. 1:200). Sections were washed and

incubated for 1 hr, at room temperature, in biotinylated swine anti-rabbit antiserum (Dakopatts, Dako A75, Copenhagen, Denmark, 1:200). The antigen signal was visualized with the Vectastain ABC kit (Vector Labs). For immunofluorescent detection, primary antibodies were rabbit anti-Drg11 (1:500), mouse anti-Lim1/2 (Developmental Studies Hybridoma Bank, University of Iowa, 1:20), guinea-pig anti-Lmx1b and Tlx3 (gift from Thomas Müller and Carmen Birchmeier, 1:1,000), rabbit anti-trkA (Chemicon, 1:1,000), rabbit anti-CGRP (Chemicon, 1:1,000), and IB4 (Sigma, 1:1,000). The antigen signal was detected by Alexa-conjugated secondary antibodies (Molecular Probes). Fluorescent samples were captured on a confocal microscope (Bio-Rad 1024). Omitting the primary antibodies resulted in a complete absence of staining in neuronal profiles. Nissl staining was performed using 0.5% crest violet for 15 min, rinsed in tap water, dehydrated, and mounted in Eukitt.

ACKNOWLEDGMENTS

The authors thank Thomas Müller, Carmen Birchmeier, and Qiufu Ma for gifts of antibodies.

REFERENCES

Caspary T, Anderson KV. 2003. Patterning cell types in the dorsal spinal cord: what the mouse mutants say. Nat Rev Neurosci 4:289–297.

Chen ZF, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ. 2001. The paired homeodomain protein *DRG11* is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. Neuron 31:59–73.

Dauger S, Pattyn A, Lofaso F, Gaultier C, Goridis C, Gallego J, Brunet J-F. 2003. *Phox2b* controls the development of peripheral chemoreceptors and afferent visceral pathways. Development 130: 6635–6642.

Ding YQ, Yin J, Xu HM, Jacquin MF, Chen ZF. 2003. Formation of whisker-related principal sensory nucleus-based lemniscal pathway requires a paired homeodomain transcription factor, *Drg11*. J Neurosci 23:7246–7254.

Fariñas I, Yoshida CK, Backus C, Reichardt LF. 1996. Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron 17:1065–1978.

Gillespie PG, Walker RG. 2001. Molecular basis of mechanosensory transduction. Nature 413:194–202.

Goulding M, Lanuza G, Sapir T, Narayan S. 2002. The formation of sensorimotor circuits. Curr Opin Neurobiol 12:508–515.

Gross MK, Dottori M, Goulding M. 2002. Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34:535–549.

Helms AW, Johnson JE. 2003. Specification of dorsal spinal cord interneurons. Curr Opin Neurobiol 13:42–49.

Jackson M, Baird JW, Cambray N, Ansell JD, Forrester LM, Graham GJ. 2002. Cloning and characterization of *Ehox*, a novel homeobox gene essential for embryonic stem cell differentiation. J Biol Chem 277:38683–38692.

Julius D, Basbaum AI. 2001. Molecular mechanisms of nociception. Nature 413: 203–210.

Lee KJ, Jessell TM. 1999. The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci 22:261–294.

Liem KF Jr, Tremml G, Jessell TM. 1997. A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91:127–138.

Light AR, Perl ER. 1979. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J Comp Neurol 186: 133–150.

Manitt C, Colicos MA, Thompson KM, Rousselle E, Peterson AC, Kennedy TE. 2001. Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. J Neurosci 21:3911–3922.

Mattise M. 2002. A dorsal elaboration in the spinal cord. Neuron 34:491–493.

McCormick LJ, Hutt JA, Hazan J, Houart C, Cohen J. 2007. The homeodomain transcription factor *drg11* is expressed in primary sensory neurons and their putative CNS targets during embryonic development of the zebrafish. Gene Expr Pattern 7:289–296.

Müller T, Brohmann H, Pierani A, Heppenstall PA, Lewis GR, Jessell TM, Birchmeier C. 2002. The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34:551–562.

Qian Y, Fritzsch B, Shirasawa S, Chen CL, Choi Y, Ma Q. 2001. Formation of brainstem noradrenergic centers and first-order relay visceral sensory neurons is dependent on homeodomain protein Rnx/Tlx3. Genes Dev 15:2553–2545.

Qian Y, Shirasawa S, Chen CL, Cheng L, Ma Q. 2002. Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1. Genes Dev 16:1220–1233.

Rebelo S, Chen ZF, Anderson DJ, Lima D. 2006. Involvement of DRG11 in the development of the primary afferent nociceptive system. Mol Cell Neurosci 33:236–246.

Reynolds ML, Fitzgerald M, Benowitz LI. 1991. GAP-43 expression in developing cutaneous and muscle nerves in the rat hindlimb. Neuroscience 41:201–211.

Saito T, Greenwood A, Sun Q, Anderson DJ. 1995. Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol Cell Neurosci 6:280–292.

Saper CB. 2000. Brain stem, reflexive behavior, and the cranial nerves. In: Kandel ER, Schwartz JH, Jessell TM, editors. Principles of neural science. New York, NY: McGraw-Hill. p 873–888.

White FA, Silos-Santiago I, Molliver DC, Nishimura M, Phillips H, Barbacid M, Snider WD. 1996. Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. J Neurosci 16:4662–4672.

Willis WD, Westlund KN, Carlton SM. 2004. Pain system. In: Paxinos G, editor. The rat nervous system. 3rd ed. Chapt. 27. Boston: Elsevier Academic Press. p 853–890.

Zimmermann M. 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109–110.

Neuron, Vol. 31, 59–73, July 19, 2001, Copyright ©2001 by Cell Press

The Paired Homeodomain Protein DRG11 Is Required for the Projection of Cutaneous Sensory Afferent Fibers to the Dorsal Spinal Cord

Zhou-Feng Chen,[1,7,9] Sandra Rebelo,[3,9] Fletcher White,[4,8] Annika B. Malmberg,[2,8,10] Hiroshi Baba,[5,11] Deolinda Lima,[3] Clifford J. Woolf,[5] Allan I. Basbaum,[2] and David J. Anderson[1,6]
[1]Division of Biology 216-76 and Howard Hughes Medical Institute California Institute of Technology Pasadena, California 91125
[2]Departments of Anatomy and Physiology and W.M. Keck Foundation Center for Integrative Neuroscience University of California, San Francisco San Francisco, California 94143
[3]Instituto de Histologia e Embriologia Universidade do Porto Porto Portugal
[4]Department of Neurology and PVA/EPVA Neuroscience Research Center Yale Medical School New Haven, Connecticut 06510 and Rehabilitation Research Center VA Medical Center West Haven, Connecticut 06516
[5]Department of Anesthesia and Critical Care Massachusetts General Hospital Harvard Medical School Boston, Massachusetts 02129

Summary

Cutaneous sensory neurons that detect noxious stimuli project to the dorsal horn of the spinal cord, while those innervating muscle stretch receptors project to the ventral horn. DRG11, a paired homeodomain transcription factor, is expressed in both the developing dorsal horn and in sensory neurons, but not in the ventral spinal cord. Mouse embryos deficient in DRG11 display abnormalities in the spatio-temporal patterning of cutaneous sensory afferent fiber projections to the dorsal, but not the ventral spinal cord, as well as defects in dorsal horn morphogenesis. These early developmental abnormalities lead, in adults, to significantly attenuated sensitivity to noxious stimuli. In contrast, locomotion and sensori-motor functions appear normal. *Drg11* is thus required for the formation of spatio-temporally appropriate projections from nociceptive sensory neurons to their central targets in the dorsal horn of the spinal cord.

[6]Correspondence: mancusog@caltech.edu
[7]To whom requests for materials should be directed.
[8]These authors contributed equally to this work.
[9]Present address: Departments of Anesthesiology, Psychiatry, Molecular Biology and Pharmacology, Washington University School of Medicine Pain Center, St. Louis, Missouri 63110.
[10]Present address: NeurogesX, Inc., San Carlos, California 94070.
[11]Present address: The Department of Anesthesia, Niigata University, Niigata, Japan.

Introduction

The accurate perception of external sensory information by the brain requires appropriate synaptic connectivity between peripheral sensory neurons of a given modality, and their central targets. How such central connectivity is coordinated with the specification of sensory modality is poorly understood, and remains a central problem in neural development. In the mammalian olfactory system, for example, a family of odorant receptors (Buck and Axel, 1991) both determines sensory modality and contributes to the specificity of topographic projections to the olfactory bulb (Wang et al., 1998). Whether such a conservative mechanism for coordinating sensory modality and connectivity is employed by other developing sensory systems is not clear, however.

Primary somatosensory neurons in mammalian dorsal root ganglia (DRG) are a heterogeneous population subserving diverse sensory modalities, including pain, touch, and body position (Scott, 1992). These different modalities are associated with topographically distinct projections to the spinal cord, the first relay station in the central nervous system (CNS). For example, large diameter muscle stretch receptor neurons project to the ventral spinal cord (Figure 1A, red), while cutaneous afferent neurons project to superficial laminae I and II of the dorsal horn (Willis and Coggeshall, 1991) (Figure 1A, blue and green). The latter population includes neurons specialized for nociception, the sensation of noxious stimuli. Low-threshold mechanosensory neurons, in turn, project to deeper layers III and IV (Brown et al., 1977; Ralston et al., 1984; Shortland et al., 1989). Thus, to a first approximation different stimulus features of primary sensory neurons are topographically mapped to different laminae of the spinal cord (Figure 1A).

The molecular mechanisms that govern the connectivity of different subsets of primary sensory neurons with their central targets are only beginning to be understood. Semaphorins and their receptors, the neuropilins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), have been proposed to control the projections of cutaneous versus muscle sensory afferent fibers into the dorsal versus ventral spinal cord based on in vitro assays (Messersmith et al., 1995; Puschel et al., 1996). However, these molecules have not yet been shown to be essential for this process in vivo (Kitsukawa et al., 1997; Chen et al., 2000; Giger et al., 2000). Similarly, in vitro studies have suggested that Slit molecules may control the initial branching of sensory afferent fibers into the spinal gray matter (Wang et al., 1999), but whether they play this role in vivo is not yet clear.

Transcription factors that control the connectivity of sensory neurons and their central targets are also beginning to be identified. For example Ets-domain transcription factors, such as ER81 and PEA3, are coordinately expressed by subsets of muscle afferent sensory neu-

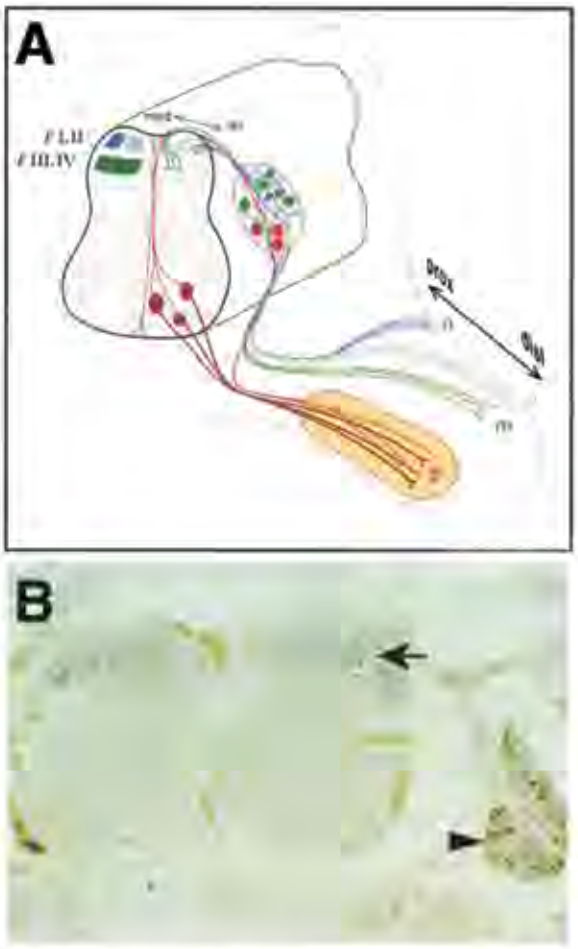

Figure 1. Organization of Primary Sensory Afferent Projections to the Spinal Cord and Relationship to Expression of DRG11

(A) Schematic diagram illustrating the organization of sensory neuron projections to the spinal cord. Muscle afferent sensory neurons (red), which mediate proprioception (p), project to motoneurons in the ventral spinal cord (magenta) and to muscle spindles in the periphery (yellow oval). Cutaneous afferents with peripheral projections in the skin project to different laminae in the dorsal spinal cord: nociceptive neurons (n, light and dark blue) to lamina I and II (*I*, II), and mechanoceptive neurons (m, green) to laminae III and IV (*I*III, IV). Within a given lamina, cutaneous afferents innervating proximal peripheral targets project to the lateral region of the dorsal horn (n, dark blue), while those with distal peripheral targets project more medially (n, light blue). For simplicity, only mechanoceptive afferents with distal targets are illustrated.

(B) Expression of DRG11 mRNA in the DRG and spinal cord at postnatal day 3 in the rat. Arrow indicates expression in neurons of the dorsal spinal cord (substantia gelatinosa), arrowhead in sensory neurons.

rons and the motoneurons to which they connect (Lin et al., 1998). Genetic analysis has indicated that ER81 is essential for the formation of such connections (Arber et al., 2000). Interestingly, all Ets-domain transcription factors thus far examined are expressed exclusively by muscle afferent sensory neurons (Lin et al., 1998; Arber et al., 2000). This suggests that other families of transcription factors may control the connectivity of cutaneous afferent sensory neurons with their central targets in the dorsal horn.

One transcription factor that is expressed by both DRG sensory neurons and neurons in the dorsal horn is a paired homeodomain protein known as DRG11 (Saito et al., 1995). Expression of DRG11 occurs first in DRG sensory neurons and approximately one day later in the dorsal horn, around the time when cutaneous afferent fibers first penetrate the spinal gray matter (Ozaki and Snider, 1997). Induction of *Drg11* in the dorsal spinal cord is controlled independently of that in the DRG (A. Greenwood, Z.-F.C. and D.J.A., unpublished data), suggesting that its coordinated expression in these pre- and post-synaptic neurons could contribute causatively to their connectivity.

Here, we have generated and analyzed mice containing a mutation in the *Drg11* gene. *Drg11*$^{-/-}$ embryos exhibit spatio-temporal abnormalities in the initial penetration of cutaneous sensory afferent fibers into the lateral-most part of the developing dorsal horn, while sparing projections, including those of muscle afferents, to more medial and ventral regions. Subsequent to this initial deficiency, later defects in cellular differentiation and survival are observed, leading in adults to a substantial loss of both neurons and cutaneous afferent fibers in the lateral-most regions of the dorsal horn. Behavioral studies show that these anatomical defects are correlated with a substantial reduction in sensitivity to various types of noxious stimuli, while locomotion and sensorimotor function appear normal. These data suggest that DRG11 is required, directly or indirectly, for the initial formation of connections between cutaneous afferent sensory neurons and their central targets.

Results

Generation of *Drg11*-Deficient Mice

A mutation in *Drg11* was produced using homologous recombination in embryonic stem (ES) cells (Ramirez-Solis et al., 1993). The design of the targeting construct deletes exons 3 and 4, which encode most of the paired-like homeodomain, the putative DNA binding region (Figures 2A and 2B). RT-PCR experiments failed to identify transcripts that encoded residual homeodomain sequences upstream of the deleted exons in *Drg11*$^{-/-}$ mice (see Experimental Procedures). Therefore, the mutation is likely to abolish DRG11 function. Targeted ES cell clones were identified by Southern blotting (Figure 2C), and germline chimeras were obtained from C57BL/6J host blastocysts injected with these clones (Hogan et al., 1986). *Drg11* heterozygous mice were viable, fertile, and apparently normal. In a mixed 129/SvJ x C57BL/6J genetic background, *Drg11*$^{-/-}$ mice were born in the expected Mendelian ratio (31 +/+: 45 +/−: 25 −/−), but weighed less than wild-type or heterozygous littermates. By about 3 weeks of age, however, all *Drg11*$^{-/-}$ mice in this genetic background had died.

Early Developmental Defects in the Dorsal Horn of *Drg11*$^{-/-}$ Embryos

We first looked for evidence of phenotypic abnormalities in *Drg11*$^{-/-}$ embryos at stages just after the gene is first expressed. In wild-type embryos, *Drg11* is expressed in the dorsal spinal cord beginning on E12-E12.5 (Figure 2E and Saito et al., [1995]). Within this region, *Drg11* is initially expressed by newly generated neurons adjacent to the ventricular zone (Figure 2E, upper small arrow),

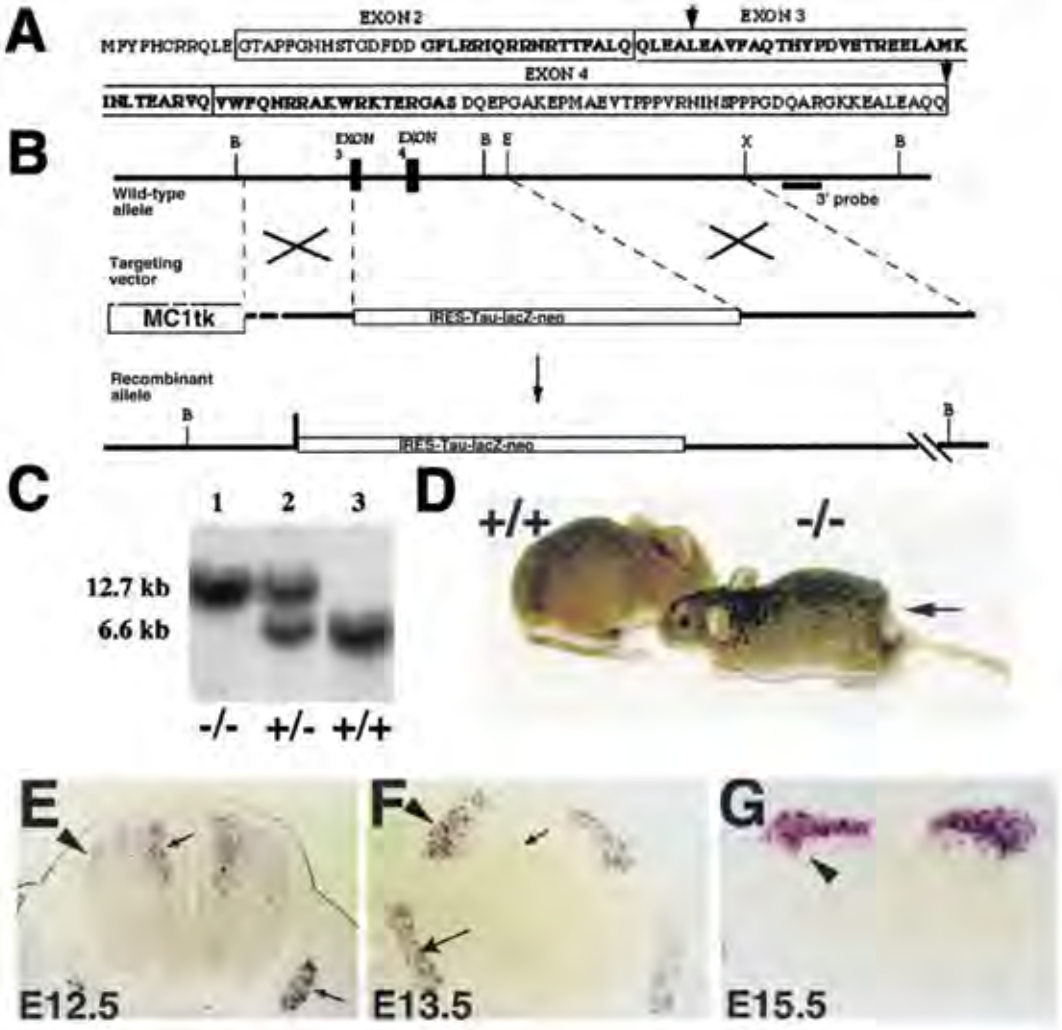

Figure 2. Targeted Mutagenesis of *Drg11* in Mice

(A) Amino acid sequence of the first coding exons (boxed). Black letters encode the homeodomain, and the arrows indicate the boundaries of the deleted region.

(B) Targeting strategy for the *Drg11* gene. Black box indicates the coding exons. Following homologous recombination, coding exons 3 and 4, which encode most of the homeodomain region, are replaced by the IRES-Tau-lacZ-neo cassette. B: BamH1.

(C) Southern blot of $Drg11^{+/-}$ intercross progeny. Wild-type (6.6 kb) and mutant (12.7 kb) alleles are distinguishable by BamH1 digestion using an 0.8kb Pst1 genomic fragment as a 3′ external probe. Lane1, homozygote (−/−); lane 2, heterozygote (+/−); lane 3, wild-type (+/+).

(D) Phenotype of a $Drg11^{-/-}$ mouse (right, −/−) and a wild-type littermate (left, +/+). Note the characteristic skin lesion on the dorsal aspect of the proximal hindlimb (arrow).

(E–G) Expression of *Drg11* mRNA in wild-type mouse embryos at E12.5 (E), E13.5 (F), and E15.5 (G). Arrowheads indicate expression in the lateral region of the dorsal horn. Upper small arrows in (E) and (F) indicate expression just outside the ventricular zone; lower small arrows indicate expression in the DRG. Expression is also detected in the DRG at E15.5, but is not shown in (G).

as well as in scattered cells lateral to this region (Figure 2E, arrowhead). Over the next few days, expression in this medial location is extinguished (Figure 2F, arrow), while it increases in the dorso-lateral region (Figures 2Fand 2G, arrowheads).

Nissl staining of E14.5 dorsal horns revealed no obvious difference between wild-type and $Drg11^{-/-}$ embryos (Figures 3A–3D). However, beginning at E15.5, several differences could be observed. First, there appeared to be a reduction in the intensity of Nissl staining in the dorsal horn of $Drg11^{-/-}$ embryos (Figures 3E–3H, arrows), in the same area where *Drg11* itself is expressed at this stage in wild-type embryos (Figure 2G, arrow). This reduction in staining intensity occurs in a region populated by small, darkly staining neurons (Figures 3G and 3H, arrows). In addition, the dorsal funiculus, which consists mainly of primary afferent fibers and second-order projections at these embryonic stages, was shallower (Figures 3E–3H, brackets, "DF"). These differences between wild-type and $Drg11^{-/-}$ dorsal horn were more pronounced at thoraco-lumbar than at cervical levels (cf. Figures 3E and 3F versus Figures 3G and 3H, arrows). No defects in dorsal horn morphology were observed in heterozygous $Drg11^{+/-}$ embryos (not shown).

Despite the clear defects visible by Nissl staining, we were unable to identify any consistent, obvious alterations in the expression of several molecular markers

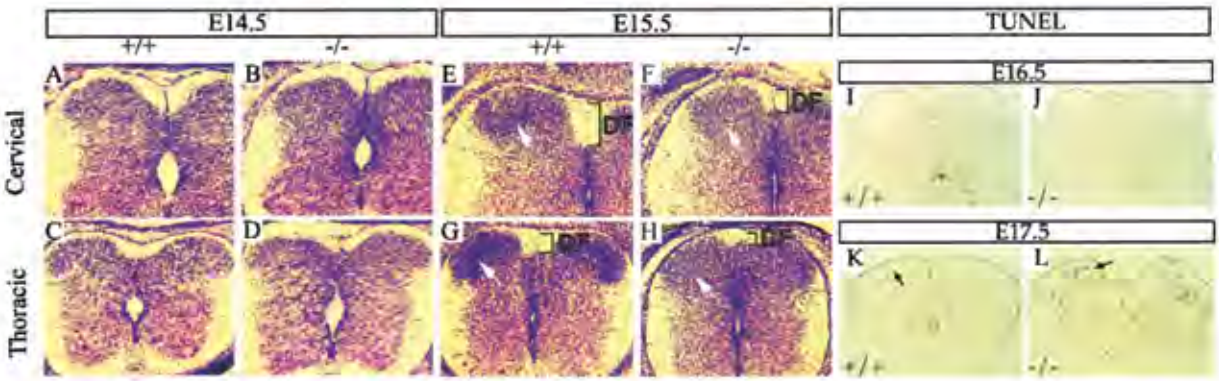

Figure 3. Timing of Morphological Abnormalities and Cell Death in the Dorsal Horn of *Drg11*$^{-/-}$ Mice

(A–H) Nissl-stained sections of E14.5 (A–D) and E15.5 (E–H) spinal cord. No difference between mutant (−/−) and wild-type (+/+) specimens are detectable at E14.5. At E15.5, reduced Nissl staining is visible in the dorsal horns of mutant embryos (F) and (H), arrows). The dorsal funiculus ("DF, brackets") is also shortened. Note that the difference between mutant and wild-type specimens is more obvious at thoracic (G) versus (H) than at cervical (E) versus (F) levels. Heterozygous embryos were indistinguishable from wild-type (not shown).

(I–L) TUNEL labeling of apoptotic cell death. Enhanced cell death in the *Drg11*$^{-/-}$ spinal cord is first detected at E17.5 (L), two days after defects are detectable by Nissl staining (F and H).

in the dorsal spinal cord of midgestational *Drg11*$^{-/-}$ embryos. These markers included the transcription factors Ebf1 and Ebf2 (Garel et al., 1997; Wang et al., 1997) (Figures 4A–4D), Lmx1b (Chen et al., 1998a) (Figures 4E and 4F), Math1 (Akazawa et al., 1995), LH2a (Xu et al., 1993; Liem et al., 1997), and Pax3 (Goulding et al., 1993)

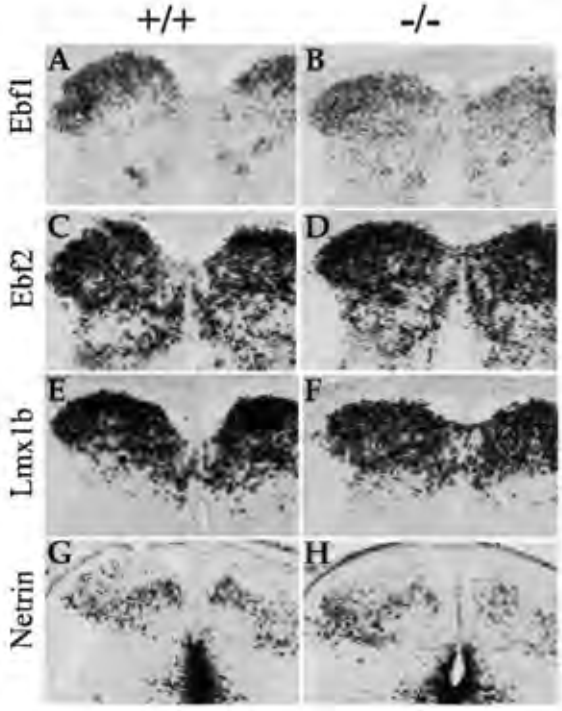

Figure 4. Expression of Molecular Markers in the Dorsal Horn of *Drg11*$^{-/-}$ Embryos

Sections through dorsal horn of wild-type (+/+) and *Drg11*$^{-/-}$ embryos (−/−) at E14.5 are shown. Three transcription factor markers, Ebf1 (A and B), Ebf2 (C and D), and Lmx1b (E and F) are shown, along with the axon guidance molecule Netrin (G and H). No differences are seen except for the slight flattening of the dorsal horn in the mutant (see also Figure 3).

(data not shown), and the axon guidance molecule Netrin 1 (Leonardo et al., 1997) (Figures 4Gand 4H).

We next asked whether the reduction in Nissl staining detected in the dorsal horn of *Drg11*$^{-/-}$ embryos was due to cell death. Prior to E17.5, there were few if any TUNEL^{+} cells in the spinal cord of *Drg11*$^{-/-}$ embryos (Figure 3J and data not shown), despite the reduced Nissl staining evident one day earlier (Figures 3F and 3H). However, beginning on E17.5, increased cell death was apparent in the dorsal horn (Figures 3K and 3L, arrows), in the same region where the decrease in Nissl staining was visible two days earlier (Figures 3G and 3H, arrows). These data suggest that the decrease in Nissl staining intensity evident at E15.5 is unlikely to be due to the death of small darkly staining neurons. More likely, it reflects a defect in some aspect of their differentiation. This defect is eventually followed by cell death, but not until two days later.

A Defect in the Projection Pattern of Primary Sensory Afferents in the Dorsal Horn of *Drg11*$^{-/-}$ Embryos

We next examined the development of primary sensory afferent projections to the embryonic dorsal horn in *Drg11*$^{-/-}$ mice. In wild-type animals, the central projections of cutaneous nociceptive sensory neurons first arrive in the dorsal root entry zone (DREZ) at E10.5, and begin to invade the spinal gray matter at E12.5 (Ozaki and Snider, 1997). Staining with antibody to calbindin-28K marks a subset of cutaneous neurons and their afferent fibers (Honda, 1995). Ingrowth of such calbindin^{+} afferent fibers into the lateral aspect of the dorsal horn occurred between E12.5 and E13.5, in wild-type embryos (Figures 5C and 5G, arrow). In contrast, no such calbindin^{+} fibers were detected in the dorsal horn of *Drg11*$^{-/-}$ embryos at E13.5 (Figure 5H, arrow). Nissl staining of adjacent sections revealed no apparent morphological defects in the developing dorsal horn at this stage (Figures 5E and 5F) or at E12.5 (Figures 5A and 5B), consistent with the analysis of E14.5 embryos (Figures 3A–3D).

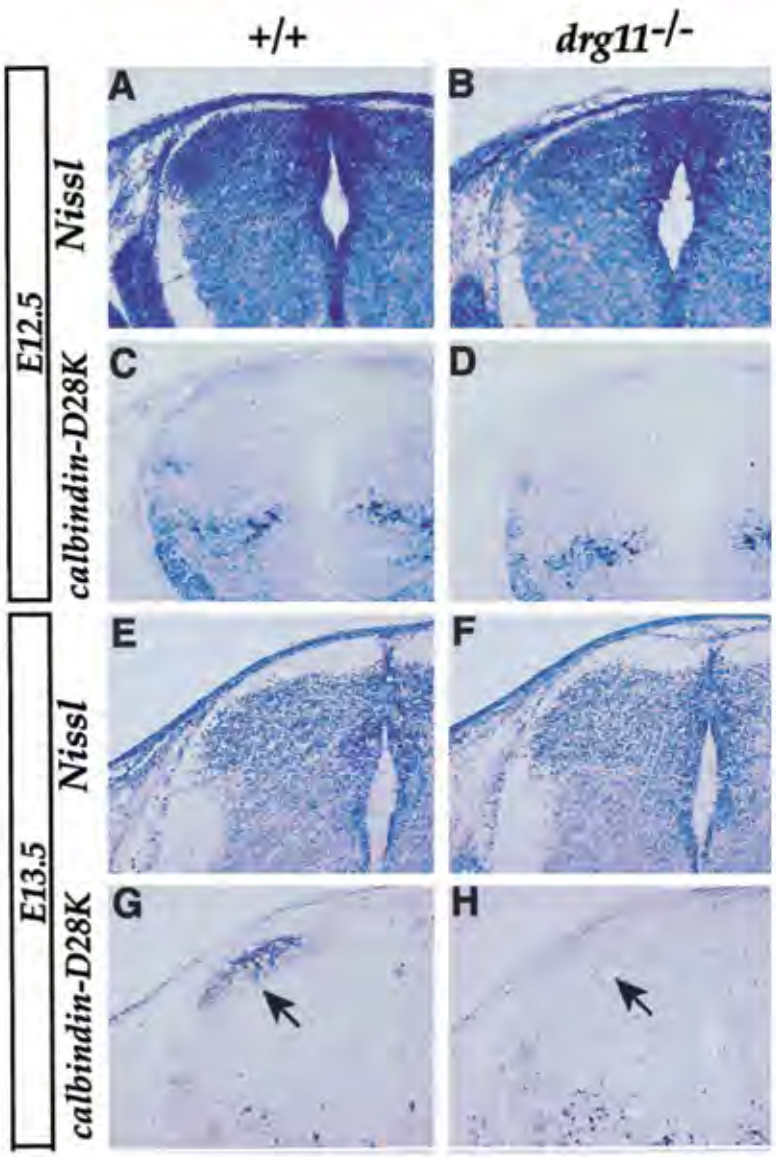

Figure 5. A Connectivity Defect Precedes Morphological Defects in *Drg11*$^{-/-}$ Embryos

([A], [C]), ([B], [D]), ([E], [G]) and ([F], [H]) are pairs of adjacent sections stained with Nissl (A, B, E, and F) and anti-calbindin-28K antibody (C, D, G, and H), respectively.

(A–D) At E12.5, no difference in either Nissl or calbindin staining is detectable between wild-type (A) and (C) and mutant (B) and (D) specimens. At this stage, calbindin^{+} sensory afferent fibers have not yet penetrated the dorsal horn gray matter.

(E–H) At E13.5, calbindin^{+} afferents are seen to penetrate the gray matter of wild-type (G, arrow) but not mutant (H, arrow) dorsal horn, although no abnormalities are yet detectable by Nissl staining (E and F). Heterozygous specimens were indistinguishable from wild-type.

To determine whether the absence of calbindin^{+} fibers at E13.5 reflected a complete block or rather a delay in cutaneous afferent fiber ingrowth, we examined later embryos. At E14.5, calbindin^{+} fibers could be seen in the DREZ of *Drg11*$^{-/-}$ embryos (Figure 6B, arrow), but had not penetrated into the spinal gray matter to the extent visible in wild-type embryos (Figure 6A, arrow). In addition, there was a marked absence of such fibers in the lateral-most portion of the dorsal horn (Figures 6A and B; arrowheads). By E16.5, calbindin^{+} fibers had invaded the dorsal horn of *Drg11*$^{-/-}$ mutants (Figure 6D). However, the absence of such fibers in the lateral-most region of the dorsal horn persisted (Figures 6C and 6D; arrowheads). At the same time, there was an increased density of calbindin^{+} fibers near the midline of *Drg11*$^{-/-}$ embryos in comparison to wild-type (Figures 6C and 6D; arrows). These data suggest that the initial absence of calbindin^{+} fibers at E13.5 (Figure 5H) reflects a delay, rather than a block, in afferent fiber ingrowth, but also indicate that the ingrowth that eventually does occur is biased toward the medial region of the dorsal horn, in comparison to wild-type embryos.

Similar results were obtained using antibodies to trkA, which is expressed by most or all cutaneous sensory neurons at this stage (Lewin, 1996; Snider and Silos-Santiago, 1996) (Figures 6E–6H). At E16.5, the lateral-to-medial shift in the distribution of trkA^{+} afferents between wild-type and *Drg11*$^{-/-}$ embryos was clear, with many more fibers appearing to cross the midline in the mutant (Figures 6G and 6H, arrows). In contrast to the abnormal cutaneous afferent projections, no differences between wild-type and *Drg11*$^{-/-}$ embryos were observed in the central projections of IA muscle afferent fibers (Figures 6I and 6J, arrows) as revealed by staining with antibodies to peripherin, which marks such proprioceptive afferents at these embryonic stages (Escurat et al., 1990; Goldstein et al., 1991).

Because apparent differences in afferent fiber projections revealed by antibody staining might reflect differences in the distribution of the corresponding antigens,

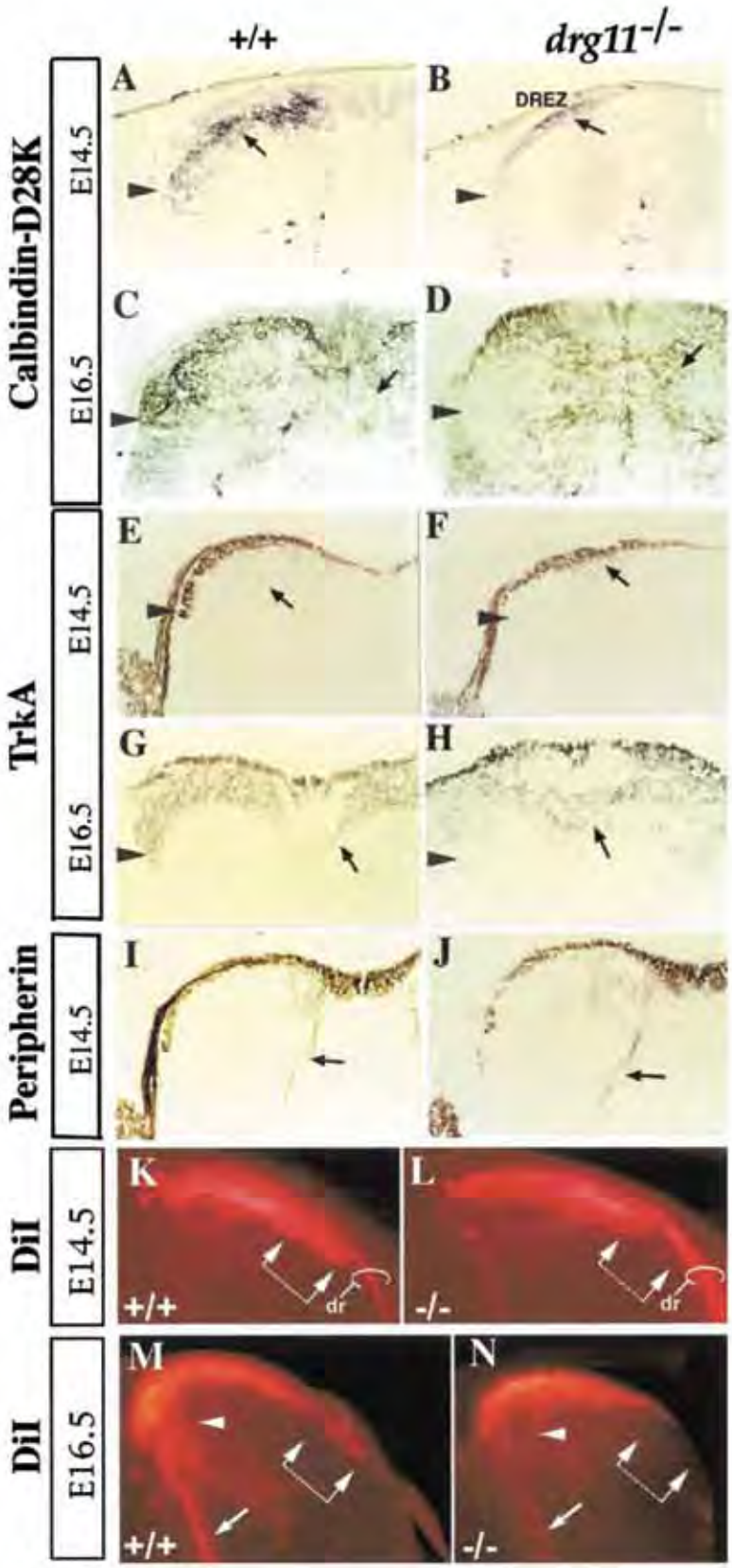

Figure 6. Developmental Progression of Afferent Projection Defects in the Dorsal Horn of *Drg11*$^{-/-}$ Mice

(A–D) Calbindin-D28K staining. Note that at E14.5 (A and B), calbindin^{+} fibers have already entered the spinal gray matter in wild-type embryos (A, arrow), while in the mutant they are restricted to the most superficial layer (B, arrow) and absent from the lateral region (cf. [A] versus [B],

rather than of the fibers that express them, we used DiI labeling as an independent assessment of afferent fiber projections in *Drg11*$^{-/-}$ and wild-type embryos (Figures 6K–6N). These studies confirmed the abnormalities in cutaneous afferent fiber projection to the dorsal horn detected by antibody staining. First, there was a virtually complete absence of afferent fiber ingrowth into the lateral-most portion of the dorsal horn, at E14.5 (Figures 6K and 6L, arrowed bracket), even though the growth of sensory axons to the DREZ through the dorsal root was unaffected (Figures 6K and 6L, dr). This defect in afferent fiber penetration to the dorso-lateral gray matter persisted at E16.5 (Figures 6M and 6N, arrowed bracket), although afferent fibers could be detected in the dorso-medial region of mutant embryos at this stage (Figures 6M and 6N, arrowheads). Consistent with the results of peripherin staining, there appeared to be no difference in the ventro-medial projections of IA muscle afferent fibers (Figures 6M and 6N, large arrows).

Taken together, these data indicate that in *Drg11*$^{-/-}$ embryos there is a defect in the spatio-temporal patterning of sensory afferent fiber projections to the dorsal horn, which selectively affects cutaneous afferents. These abnormalities are especially prominent in the lateral-most region of the dorsal horn, a site where expression of *Drg11* itself is most abundant at this stage (Figure 2F, arrowhead). This is also the region where defects in Nissl staining are eventually apparent, at E15.5 (Figures 3E–3H, arrows). However, the stage when the cutaneous afferent fiber projection defect is first detected (E13.5; Figures 5G and 5H) precedes the abnormalities in Nissl staining by two days. No defects in afferent fiber projections were detectable in *Drg11*$^{+/-}$ heterozygous mice, at any stage examined (data not shown).

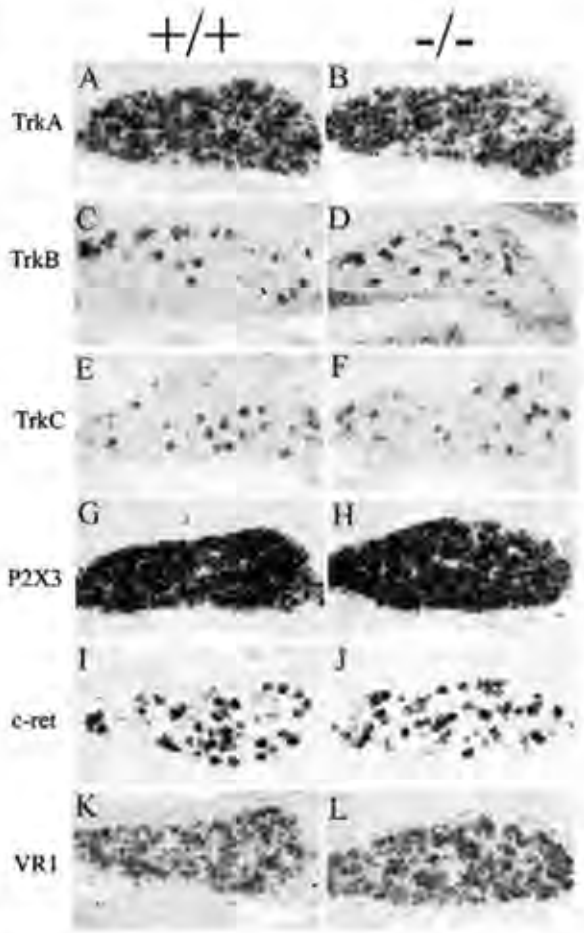

Figure 7. Expression of Sensory Neuron Markers Appears Normal in the DRG of *Drg11*$^{-/-}$ Mice

A series of adjacent sections through E14.5 lumbar DRG of wild-type and *Drg11* mutant embryos is shown. All photomicrographs are nonisotopic in situ hybridizations performed with the probes indicated to the left of the photomicrographs. No difference in the expression of any of the markers is detected.

Differentiation and Survival of DRG Sensory Neurons Are Normal in *Drg11*$^{-/-}$ Embryos and Neonates

DRG11 is also expressed in developing sensory neurons of the dorsal root ganglia (DRG), at the same time as it is detected in the dorsal horn (Figures 2E and 2F, lower arrows). At E14.5, no differences between wild-type and *Drg11*$^{-/-}$ embryos were detectable using a battery of molecular markers for different sensory neuron subtypes (Figure 7). The total number of sensory neurons was also not significantly different in lumbar (L3-L5) DRG between wild-type (18,943 ± 1,437; mean ± SEM) and *Drg11*$^{-/-}$ mutant ganglia (16,334 ± 713; $p = 0.193$; $n = 2$ independent *Drg11*$^{-/-}$ mutant and wild-type littermate embryos counted). Consistent with these results, there was no increase in TUNEL labeling in the DRG of *Drg11*$^{-/-}$ mice, even at a stage (E17.5) when TUNEL^{+} cells were apparent in the dorsal horn (Figure 3L; data not shown). To determine whether any loss of cutaneous afferent sensory neurons occurred perinatally, we also quantified the number of trkA^{+} sensory neurons in neo-

arrowheads). At E16.5 (C and D), calbindin staining is most intense in presumptive laminae I and II at the lateral margins (C, arrowhead). In the mutant (D), calbindin-positive fibers have penetrated the gray matter by this stage, but are more concentrated in the medial region (arrow) and depleted from the lateral region (D, arrowhead).

(E–H) trkA antibody staining. The entry of trkA^{+} afferent fibers into the gray matter of *Drg11* mutant mice is delayed relative to wild-type (E and F, arrows). It is also biased toward the medial region (F and H, arrows) and depleted from the lateral region (F and H, arrowheads).

(I and J) Peripherin staining reveals no difference between mutant (J) and wild-type (I) in the ingrowth of group IA muscle sensory afferents that grow to the ventral spinal cord (arrows).

(K–N) DiI labeling of lumbar level sensory afferents in the sciatic nerve. At E14.5 (K and L), afferent ingrowth to the lateral dorsal horn is apparent in wild-type (K, arrowed bracket), but not in *Drg11*$^{-/-}$ specimens (L, arrowed bracket). Nevertheless, sensory afferent fibers appear to grow normally to the dorsal roots (dr) in the mutant. By E16.5 (M and N), afferents in the mutant have entered the gray matter and are present in the medial region of the gray matter (N, arrowhead), but are still absent from the lateral region (N, arrowed bracket). Presumptive IA muscle afferent projections (arrows) are unaffected, consistent with the results of anti-peripherin antibody staining (I and J).

Table 1. Number of trkA$^+$ Neurons in Lumbar DRG of P0 *Drg11*$^{-/-}$ Mice

Ganglion	+/+	*Drg11* −/−	t test
L2	1230 ± 156	1348 ± 247	NS
L3	1634 ± 54	1723 ± 65	NS
L4	1972 ± 58	2306 ± 450	NS
L5	2344 ± 0	2044 ± 393	NS

Numbers indicate the sum of the neurons counted in the right plus left ganglia at each of the indicated axial levels. Data represent the mean ± SEM from two separate mutant and wild-type embryos. Numbers are not corrected. NS, not significantly different ($p > 0.05$).

natal *Drg11*$^{-/-}$ mice (Table 1). Again, there was no significant difference in the number of these neurons in L2-L5 DRG between wild-type and *Drg11*$^{-/-}$ mutants.

Absence of PKCγ$^+$ Cells in Lamina IIi of Early Postnatal *Drg11*$^{-/-}$ Mice

The abnormal Nissl staining in the dorsal horn of E15.5 *Drg11*$^{-/-}$ embryos (Figures 3E–3H) suggested that the mutation might affect the development of second-order neurons involved in the central processing of nociceptive afferent input to the spinal cord. One marker of such neurons is protein kinase C-γ (PCKγ), which is specifically expressed in lamina IIi and has been functionally implicated in nerve injury-induced ("neuropathic") pain (Malmberg et al., 1997). In wild-type mice, PKCγ is first expressed in the dorsal horn at postnatal day 2 (P2; data not shown). Strikingly, in postnatal day 5 (P5) *Drg11*$^{-/-}$ mice, there was a virtually complete absence of PKCγ$^+$ cells across the entire medio-lateral extent of the dorsal horn (Figure 8C, arrow, versus Figure 8D). The expression of this marker in the corticospinal tract, however, was unchanged (Figure 8D, large arrow), providing an internal positive control for the staining. The lack of PKCγ expression could also be observed at P2 (not shown). The absence of PKCγ$^+$ cells is not due simply to generalized neuronal death, because many neurons in the dorsal horn were still present at this stage as indicated by staining for the pan-neuronal nuclear marker NeuN (Figures 8A and 8B), as well as for other dorsal horn markers (not shown). However, we cannot distinguish whether the loss of this specific marker reflects selective cell death or, rather, defective differentiation.

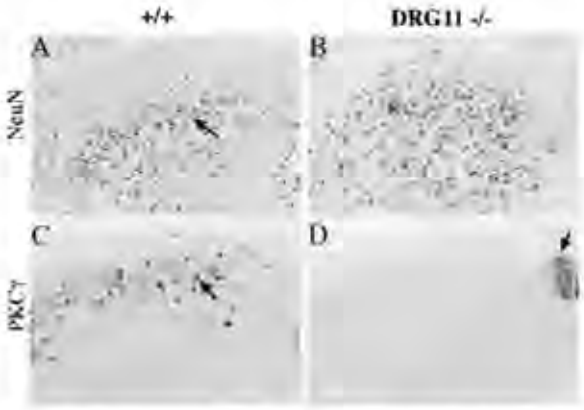

Figure 8. Early Loss of PKCγ Expression in Lamina IIi of the Dorsal Horn

(A and B) NeuN staining of neuronal cell bodies reveals a disorganization of the dorsal horn in the mutant at postnatal day 5 (B).

(D) PKCγ staining is eliminated in lamina IIi of the mutant (D), although it is retained in corticospinal axons in the dorsal funiculus (D, arrow). Similar results were also obtained at P2 (not shown). Note that the loss of PKCγ$^+$ cells is relatively selective, in that there are still many surviving neurons in the dorsal spinal cord at this stage (B).

Defects in Pain Sensitivity in Adult *Drg11*$^{-/-}$ Mice

The foregoing data suggested that the *Drg11*$^{-/-}$ mutation selectively perturbs the development of primary cutaneous sensory afferent projections to the dorsal horn (as well as subsequent development of the dorsal horn itself). As these projections mediate (among other modalities) nociception, these results implied that *Drg11*$^{-/-}$ mice might exhibit selective deficiencies in their behavioral responses to noxious stimuli. Because *Drg11*$^{-/-}$ mice die by the third postnatal week in a 129Sv x C57BL/6J background, we attempted to extend their lifespan by crossing them to CD-1 mice, which are a more vigorous outbred strain. In this mixed genetic background, about 50% of *Drg11*$^{-/-}$ mice did survive to adulthood. Nevertheless, analysis of *Drg11*$^{-/-}$ embryos in this mixed background revealed similar developmental defects as described previously for the 129Sv x C57BL/6J background (data not shown).

At the age of three or four weeks, there was no difference in normal, spontaneous behavior between the *Drg11*$^{-/-}$ and wild-type mice. However, by about two months of age, *Drg11*$^{-/-}$ mice on the outbred CD-1 background could be recognized by persistent grooming of the dorsal hindlimb, which subsequently led to fur loss and skin lesions on some of the mice (Figure 2D, arrow). Such bare patches are observed in more anterior regions of mice whose cutaneous sensory neurons have been destroyed by capsaicin treatment (Crowley et al., 1994; Smeyne et al., 1994; Thomas et al., 1994). Their location in the posterior region of adult *Drg11*$^{-/-}$ mice is consistent with the fact that the developmental phenotype appears more severe caudally than rostrally and is therefore suggestive of compromised nociception in these mutants.

To experimentally assess nociceptive function, we performed a battery of behavioral tests on adult *Drg11*$^{-/-}$ mice (Figure 9). In virtually all of these tests, there was a clear and statistically significant reduction in the response of *Drg11*$^{-/-}$ mice to a variety of noxious stimuli applied to the hindpaw or tail, relative to both wild-type and *Drg11*$^{+/-}$ heterozygous littermate controls. For example, *Drg11*$^{-/-}$ mice displayed significantly higher response latencies in the hot plate, tail-flick, and paw withdrawal tests of thermal sensitivity (Figure 9A and data not shown). *Drg11*$^{-/-}$ mice also showed reduced sensitivity to mechanical stimulation, tested using using von Frey filaments (Chaplan et al., 1994) (Figure 9B). In addition, they exhibited reduced responses in tests of chemical nociception, using either formalin or capsaicin (Figure 9C). No significant differences were observed between wild-type and *Drg11*$^{+/-}$ heterozygous mice in any of these tests ($n = 4$ *Drg11*$^{+/-}$ animals examined in each of the assays; data not shown).

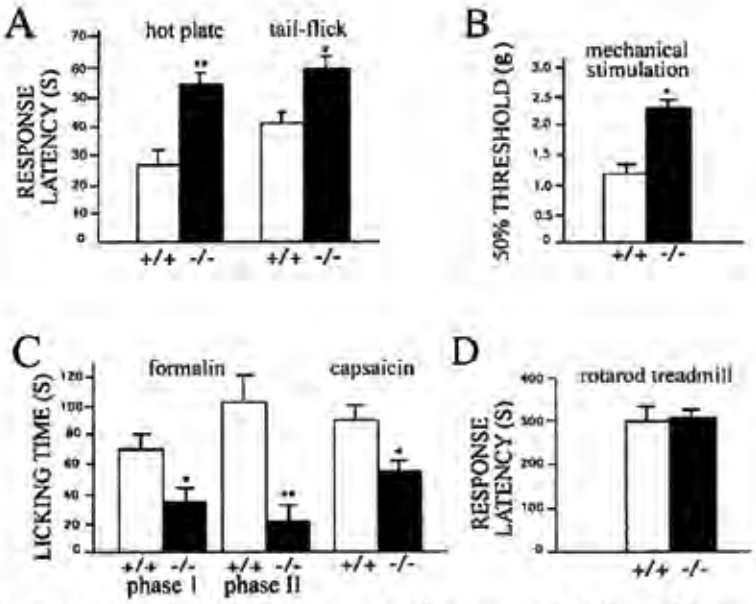

Figure 9. Reduced Sensitivity to Nociceptive and Mechanical Stimuli in *Drg11*-Deficient Mice

(A) Tests of thermal sensitivity. In both the hot plate and tail-flick tests, the response latency of *Drg11*$^{-/-}$ mice is significantly longer (**, $p < 0.001$; *, $p < 0.01$; t test) than that of wild-type littermates.

(B) Test of sensitivity to innocuous mechanical stimulation using calibrated von Frey filaments. The threshold weight (in grams) necessary to produce a response is significantly higher (*, $p < 0.001$; Mann-Whitney test) for the *Drg11*$^{-/-}$ mutant mice.

(C) Tests of chemical nociception. Injection of a 5% formalin solution into the paw evokes two phases of paw licking: phase I reflects direct activation of primary nociceptors; phase II reflects peripheral inflammation and sensitization of dorsal horn neurons. Both phases of licking are significantly reduced (*, $p < 0.05$; **, $p < 0.01$, t test) in the mutant. Licking responses to capsaicin injection into the hindpaw are also significantly reduced (*, $p < 0.05$, t test). In (A) through (C), n = 8 wild-type and n = 7 mutant animals were examined. No differences were observed between wild-type and heterozygous *Drg11*$^{+/-}$ animals in any of these assays (not shown).

(D) Sensorimotor (IA afferent) function as determined by a rotarod treadmill test is not significantly different between wild-type and mutant animals.

Taken together, these results indicate that *Drg11*$^{-/-}$ mice exhibit significant reductions in their responses to a broad range of noxious stimuli encompassing several modalities, as well as sensitivity to mechanical stimuli. In contrast, a rotarod treadmill test revealed no difference in motor function between *Drg11*$^{-/-}$ and wild-type mice (Figure 9D), suggesting that sensorimotor functions mediated by muscle afferent sensory neurons innervating spindle fibers and Golgi tendon organs are intact. Such a conclusion is consistent with the observation that the projections of IA muscle afferent sensory neurons to the ventral spinal cord develop normally in the mutant (Figures 6I–6N). Furthermore, *Drg11*$^{-/-}$ mice exhibited normal locomotion, in contrast to mice bearing mutations in genes required for proprioceptive sensory neuron development or survival, in which hindlimb locomotion is clearly impaired (Ernfors et al., 1994; Fariñas et al., 1994; Klein et al., 1994; Arber et al., 2000). Thus, the developmental defects caused by the *Drg11*$^{-/-}$ mutation lead to functional deficits involving somatosensory functions processed by the dorsal spinal cord.

Neuronal Loss in the Superficial Laminae of the Dorsal Horn of Adult *Drg11*$^{-/-}$ Mice

To determine whether the developmental defects in *Drg11*$^{-/-}$ embryos led to persistent anatomical and/or molecular deficiencies in the adult spinal cord, which might account for the behavioral phenotype, we first examined the expression of NeuN (Figures 10A and 10B; arrowheads), a general neuronal marker (Mullen et al., 1992). Staining with anti-NeuN antibody revealed a striking loss of neurons in the dorsal horn, particularly in the lateral-most region (Figures 10 A and 10B, arrowhead), the site where the ingrowth of cutaneous afferent fibers is blocked in embryos (Figure 6). Cell counts indicated a reduction of 60%–70% of such NeuN^{+} dorsal horn neurons at lumbar levels, the region where the phenotype was most pronounced (as was observed in embryos). These anatomical defects in the lumbar dorsal horn may account for the localization of skin lesions to the posterior region of *Drg11*$^{-/-}$ animals (Figure 2D). By contrast, there was no evident loss of neurons or alteration of morphology in the ventral spinal cord, where motoneurons are located (data not shown).

To determine the molecular identity of the dorsal horn neurons that were lost, we applied a battery of reagents that mark the postsynaptic targets of cutaneous afferent projections in laminae I and II of the dorsal horn. There was a complete absence of PKCγ^{+} cells in lamina IIi in adult *Drg11*$^{-/-}$ mice (Figure 10H, arrowhead). Thus, the absence of this marker at early postnatal stages (Figure 8) does not simply reflect delayed differentiation of these cells. In addition, *Drg11*$^{-/-}$ mice exhibited a lack of expression of calretinin, a calcium binding protein expressed in laminae I and II (Ren et al., 1993) (Figures 10C and 10D; arrows). Expression of PKCβII, a protein kinase C isoform (Malmberg et al., 1997), was also virtually completely eliminated at the lateral-most margins of the dorsal horn, and was greatly reduced in more dorso-medial locations (Figures 10E and 10F; arrows). Taken together, these data reveal a pronounced loss in adult *Drg11*$^{-/-}$ mice of post-synaptic neurons in laminae I, IIo, and IIi, especially in the lateral-most region of the dorsal horn.

A Central Projection Defect in the Spinal Cord of Adult *Drg11*$^{-/-}$ Mice

We next examined the expression of markers of presynaptic cutaneous sensory afferent fibers in the dorsal horn. The dorsal horn is innervated in distinct topographic locations by two different classes of nociceptive afferent fibers. "Peptidergic" C- and Aδ fibers, con-

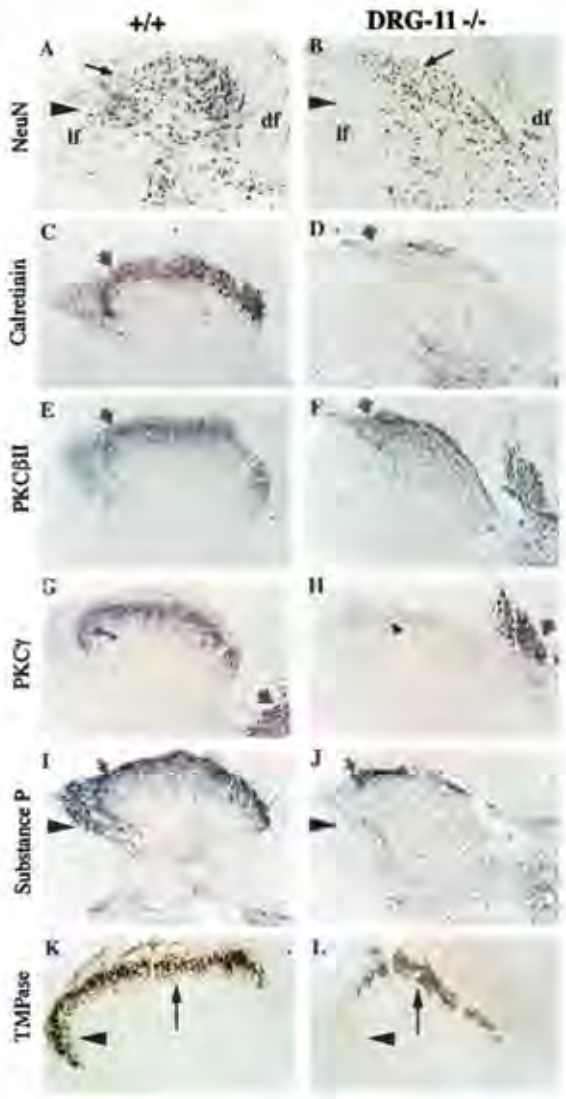

Figure 10. Loss of Dorsal Horn Neurons and Primary Afferent Projections in the Spinal Cord of Adult *Drg11*$^{-/-}$ Mice

(A and B) Staining with the pan-neuronal nuclear marker NeuN reveals abnormal morphology and neuron loss in the dorsal horn of *Drg11*$^{-/-}$ (−/−, B) mice. Note that the size of the dorsal horn of the mutant is greatly reduced, and that cell loss is particularly pronounced in the ventrolateral margin of the laminae I and II (arrowheads). lf, lateral funiculus; df, dorsal funiculus.

(C and D) Expression of calretinin in laminae I and II of the dorsal horn is virtually eliminated in the mutant (D).

(E and F) Expression of PKCβII in the dorsal horn (laminae I and II) of the mutant (F) is greatly reduced.

(G and H) Expression of PKCγ, a marker of inner lamina II (G, curved arrow) is lost in the mutant (H, arrowhead), although expression in corticospinal axons is retained in the dorsal funiculus (broad arrows).

(I and J) Cutaneous nociceptive afferent fibers identified by Substance P immunoreactivity are reduced in number and shifted dorsomedially in the mutant (J, arrow).

(K and L) Nonpeptidergic cutaneous afferent fibers, identified by expression of TMPase, which project primarily to lamina IIi, are also reduced in the mutant (L). Note the pronounced loss of afferent fibers in the lateral half of laminae I and II (I–L, arrowheads), the region of maximal cell loss (A and B). The dorsal shift of the TMPase-positive fibers (L) was confirmed by double-labeling for IB4 and CGRP (not shown) and is consistent with the loss of their normal postsynaptic targets in lamina IIi (H).

taining the neuropeptides CGRP and/or Substance P, project mainly to lamina I and outer lamina II (IIo) (Hunt et al., 1992; Snider and McMahon, 1998). There was a dramatic reduction of Substance P^{-} fibers (Figures 10I and 10J), as well as of fibers expressing CGRP (not shown), in the adult *Drg11*$^{-/-}$ spinal cord. As was the case in embryos, the lateral-most projection field of these cutaneous afferent fibers appeared almost completely eliminated (Figures 10I and 10J, arrowheads), so that there was an apparent dorso-medial shift in the distribution of surviving fibers in the mutant (Figure 10J, arrow).

The second class of nociceptive fibers are nonpeptidergic afferents expressing the surface lectin IB4, the GDNF receptor c-RET, and thiamine monophosphatase (TMPase) (Knyihar-Csillik et al., 1986; Molliver et al., 1997; Bennett et al., 1998). These afferents project specifically to lamina IIi (reviewed in Snider and McMahon (1998). As was observed for the peptidergic fibers, there was a dramatic loss of TMPase-positive fibers in the lateral-most domain of the *Drg11*$^{-/-}$ dorsal horn (Figures 10K and 10L, arrowheads). Furthermore, the remaining fibers appeared to be shifted dorso-medially, overlapping the domain occupied by SubP^{-} fibers in the mutant (Figures 10J and 10L, arrows). Double-labeling with fluorescent IB4 and anti-CGRP antibody confirmed an overlap of these peptidergic and non-peptidergic fibers in the dorso-medial region of *Drg11*$^{-/-}$ mice, rather than the well-separated staining in laminae IIi and I seen in wild-type specimens (data not shown). Interestingly, these nonpeptidergic fibers normally synapse onto PKCγ-expressing cells in lamina IIi (Snider and Wright, 1996), which are absent in the mutant (Figure 10H, arrowhead). Thus, the concentration of residual peptidergic and nonpeptidergic afferents fibers in the dorso-medial region of the *Drg11*$^{-/-}$ spinal cord may reflect the loss of their postsynaptic targets in the lateral dorsal horn, as well as throughout lamina IIi, so that the remaining fibers project to the sites where surviving intrinsic dorsal horn neurons are located.

In preliminary experiments, we sought to determine whether there were any obvious defects in synaptic transmission in the dorsal horn of adult *Drg11*$^{-/-}$ mice. Whole cell patch-clamp recordings from postsynaptic neurons (n = 14) were performed in the medial third of lamina II in slices of dorsal horn with an attached dorsal root (Baba et al., 1999). All neurons tested responded to orthodromic dorsal root stimulation and exhibited either or both monosynaptic (n = 6) or/and polysynaptic (n = 11) Aδ fiber-mediated EPSCs with short latencies (2–5ms) (Yoshimura and Jessell, 1989). In eight of fourteen lamina II neurons, C fiber-evoked long-latency (~20 ms) EPSCs were also observed at an appropriate stimulus intensity (>200 μA, 0.5 ms) (see supplemental data at http://www.neuron.org/cgi/content/full/31/1/59/DC1). The response properties of these lamina II neurons in *Drg11*$^{-/-}$ mice are similar to those extensively characterized in prior studies of normal animals (Yoshimura and Jessell, 1989; Yoshimura and Nishi, 1993; Yoshimura and Nishi, 1995; Baba et al., 1999). These data suggest that the surviving primary nociceptive afferent synapses in the dorsal horn of *Drg11*$^{-/-}$ mice function relatively normally, although more subtle defects may

Table 2. Numbers of Sensory Neurons in Lumbar DRG of Wild-Type and *Drg11* Mutant Mice

Genotype	Specimen	Nissl	trkA$^+$	CGRP$^+$	trkA/Nissl (%)	CGRP/Nissl (%)
+/+	1	9256	4861	3086	53	33
	2	12039	5448	2941	45	24
	3	9338	4744	3111	51	33
	4	9028	5220	3015	58	33
	Avg. ± SD	9915 ± 1422	5068 ± 324	3038 ± 77	52 ± 5.37	31 ± 4.5
Drg11$^{-/-}$	1	7925	3490	1935	44	24
	2	9176	3055	2141	33	23
	3	5176	2631	1973	51	38
	4	6017	3364	1869	56	31
	Avg. ± SD	7073 ± 1813	3135 ± 382	1979 ± 116	46 ± 9.97	29 ± 6.97
	t test p <	0.05	0.0003	1.6×10^{-5}	NS	NS

NS, not significantly different.

have been missed due to the limited number of neurons sampled.

Cell Loss in the DRG of Adult *Drg11*$^{-/-}$ Mice

Given the reduced number of cutaneous afferent fibers in the dorsal horn, we examined the number and phenotype of sensory neurons in adult *Drg11*$^{-/-}$ mice as well. There was a statistically significant ($p < 0.05$) reduction of almost 30% in the total number of Nissl-stained neurons in L3+L4 DRG (Table 2, Nissl). The number of nociceptive trkA$^+$ or CGRP$^+$ neurons was also reduced in *Drg11*$^{-/-}$ animals by about 30% (Table 2). However, the proportion of DRG neurons expressing these markers was unchanged (Table 2), suggestive of generalized sensory neuron loss. Consistent with this, the frequency distribution of different neuronal cell body diameters, which are characteristic of different DRG neuronal subpopulations (Scott, 1992), did not differ significantly between *Drg11*$^{-/-}$ mice and controls (see Supplementary Data). These data suggest that there is a loss of sensory neurons in adult *Drg11*$^{-/-}$ mice affecting both nociceptive and other classes of neurons. Nevertheless, as nociceptors constitute about 70% of the adult sensory neuron population, most of the total neuronal loss in *Drg11*$^{-/-}$ DRG reflects a diminution in this population.

Discussion

We have analyzed the phenotypic consequences of a mutation in *Drg11*, a paired homeodomain transcription factor that is expressed from early stages of development in both the dorsal horn and in DRG sensory neurons. Embryos deficient in DRG11 display abnormalities in the timing and position of the initial ingrowth of sensory afferent fibers to the dorsal horn and, subsequently, in morphogenesis of the dorsal horn itself. These early defects perturb the development of circuits that process nociceptive and other cutaneous sensory stimuli, as confirmed by behavioral studies in adult *Drg11*$^{-/-}$ mice. *Drg11* is thus one of the few genes to be described whose function in vivo is essential for the initial stages of assembly of the neural pathways that detect noxious stimuli.

Genetic Control of Nociceptive Circuit Formation

The formation of the neural circuits that mediate pain sensation is an important subject in neural development, yet remarkably little is known about the molecular mechanisms that control this process in vivo. The only published mutations that affect the development of nociceptors are those in the genes encoding NGF (Crowley et al., 1994), its receptor trkA (Smeyne et al., 1994), the bHLH transcription factor NGN1 (Ma et al., 1999), and the POU-domain transcription factor Brn3.0 (McEvilly et al., 1996; Xiang et al., 1996). None of these mutations, however, affects the initial establishment of connections between the DRG and the dorsal horn. NGF and trkA are required for the survival of sensory neurons long after they have differentiated and extended axons to their targets, while Brn3.0 appears to control the expression of neurotrophin receptors (Huang et al., 1999). NGN1, by contrast, controls the initial determination of trkA$^+$ sensory neuron precursors (Ma et al., 1999). Thus, the early projection defect seen in *Drg11*$^{-/-}$ embryos is distinct from other mutations affecting the development of nociceptive circuits, and may provide a useful point of entry for studies of the cell-intrinsic control of this process.

Timing and Cellular Locus of the Primary Defect in *Drg11*$^{-/-}$ Mice

The earliest detectable cellular defect in *Drg11*$^{-/-}$ mice is an abnormal projection of primary sensory afferent fibers to the dorsal horn, at E13.5. Because *Drg11* is expressed in the sensory ganglia and spinal cord at this stage, it is not clear whether this initial projection defect reflects an intrinsic function for the gene in the DRG, the dorsal horn, or both. However, the severity of the projection defect, as detected by calbindin-28K staining, appears similar at cervical and thoraco-lumbar levels (data not shown), while the subsequent defects in dorsal horn development are more prominent caudally. These observations suggest that the morphological abnormalities in the dorsal horn may develop independently of the projection defect. Consistent with this, similar morphological abnormalities, including a reduction in small, darkly staining neurons and a shortening

of the dorsal funiculus, are seen in mutants lacking *Lmx1b*, a LIM homeodomain transcription factor (Chen et al., 1998a) expressed in the dorsal horn but not in sensory neurons. In *Lmx1b*$^{-/-}$ embryos, expression of *Drg11* is lost in the spinal cord but not in the DRG (A. Kania and T.M. Jessell, personal communication). This observation supports the idea that the dorsal horn defects in *Drg11*$^{-/-}$ mice may reflect an intrinsic function for the gene in the spinal cord. Whether the projection defect reflects, conversely, an intrinsic role for DRG11 in sensory neurons or, rather, a requirement in the dorsal horn that is independent of axial position will require site-specific knockouts of *Drg11*.

DRG11 Is Required for the Proper Spatial Patterning as Well as the Timing of Cutaneous Afferent Ingrowth to the Dorsal Horn

The absence of afferent fiber ingrowth to the dorsal horn in E13.5 *Drg11*$^{-/-}$ embryos reflects a delay and not a total arrest: by E16.5 calbindin^{+} and trkA^{+} fibers have penetrated the spinal gray matter. However the increased density of these fibers medially, and increased frequency of midline crossing, suggests that the abnormal trajectory reflects more than a simple deletion of afferent projections to the lateral-most dorsal horn. Rather, both the timing and the spatial distribution of cutaneous afferent projections into the spinal gray matter are abnormal in *Drg11*$^{-/-}$ embryos.

The apparent lateral-to-medial shift in the distribution of cutaneous afferents in *Drg11*$^{-/-}$ mice may reflect alterations in the somatotopic organization of these projections. Cutaneous afferents with distal (or ventral) peripheral targets project to more medial regions of the dorsal horn, while those with more proximal (or dorsal) peripheral targets project laterally (Figure 1A; reviewed in Wilson and Kitchener [1996]). This medio-lateral somatotopy is already evident from the earliest stages of afferent fiber penetration to the dorsal horn (Mirnics and Koerber, 1995; Silos-Santiago et al., 1995). The fact that *Drg11* is expressed more abundantly in the lateral than in the medial dorsal horn (Figure 2F), taken together with the apparent medial bias of afferent fibers in the mutant, suggests that the gene may be involved in some aspect of medio-lateral patterning that underlies such somatotopy. However, it is important to note that the loss of PKCγ^{+} neurons in *Drg11*$^{-/-}$ mice was observed throughout the medio-lateral extent of lamina IIi. This may explain why deficiencies in pain sensitivity were detected distally as well as proximally in adult *Drg11*$^{-/-}$ mice.

Relationship of the Embryonic Defects in *Drg11*$^{-/-}$ Mice to the Adult Behavioral Phenotype

Behavioral tests in adult *Drg11*$^{-/-}$ animals revealed a significantly reduced sensitivity to noxious stimuli across a broad range of modalities, including mechano-, thermo-, and chemo-sensitivities. By contrast, locomotion and sensorimotor function appeared normal. Consistent with this behavioral data, we observed a dramatic cell loss in the lateral regions of the dorsal horn, as well as a reduction in afferent innervation in laminae I and II which primarily represents C- and Aδ fibers. Given this neuronal and afferent fiber loss, it is somewhat surprising that the reduction of pain sensitivity in adult *Drg11*$^{-/-}$ mice is not more complete. However, because the remaining afferent fibers mediate apparently normal synaptic transmission with their surviving second-order targets, the incomplete loss of sensitivity to noxious stimuli may simply reflect a reduced volume of synaptic information transmitted in the dorsal horn of *Drg11*$^{-/-}$ mice.

What is the connection between the early developmental defects observed in *Drg11*$^{-/-}$ embryos and the anatomical deficiencies seen in adults? The cell and afferent fiber loss in the lateral dorsal horn of adult *Drg11*$^{-/-}$ mice are consistent with the pattern of defects seen in embryos. The more general loss of PKCγ^{+} neurons may, however, reflect an independent, later action of DRG11 to control the differentiation or survival of these cells. In contrast to these embryonic and perinatal defects, the loss of sensory neurons is only observed in adult DRG. This suggests either that this sensory neuron deficit is secondary to the earlier defects or that *Drg11* has a later, independent function in these peripheral neurons.

Although the adult behavioral phenotype of *Drg11*$^{-/-}$ mice is specific to modalities mediated by cutaneous afferent sensory neurons, the neuron loss in adult DRG does not appear to be specific for this subset. While this cellular phenotype is consistent with the fact that DRG11 is expressed in trkA^{-} as well as trkA^{+} sensory neurons (Saito et al., 1995), it might appear inconsistent with the behavioral deficiencies. One possible explanation for this paradox is that the behavioral deficits may primarily reflect the combined effects of dorsal horn cell loss and afferent projection abnormalities, rather than the loss of DRG neurons per se. Consistent with this idea, defects in nociception are seen in perinatal *Drg11*$^{-/-}$ mice (Z.F.C., S.R. and D.J.A., unpublished data), an age at which the dorsal horn and afferent projection defects are apparent, but when there is not yet any detectable loss of DRG neurons (Table 1). Accordingly, the relatively modest reduction in propriospinal sensory neurons may be insufficient to cause detectable abnormalities in sensorimotor function in the absence of a corresponding central and afferent projection defect. Nevertheless, we cannot exclude that the loss of propriospinal neurons reflects an autonomous function for DRG11 in these cells that causes sensorimotor deficiencies not detected by our behavioral assays.

The Role of Transcriptional Matching in the Control of Somatosensory Circuit Formation

DRG11 is one of a relatively small number of transcription factors that are expressed in both peripheral sensory neurons and their central synaptic targets (Saito et al., 1995). Other genes with this property include *Er81*, *Phox2a/b*, and *Tlx1/3* (Tiveron et al., 1996; Lin et al., 1998; Logan et al., 1998). Such coordinated expression is highly suggestive of a functional role for these factors in controlling connectivity. Mutations in *Phox2a* and *Phox2b* primarily disrupt neuronal differentiation, however (Morin et al., 1997; Pattyn et al., 1999). In contrast, targeted disruption of *Er81* (Arber et al., 2000), like that of *Drg11*, indeed perturbs the proper formation of connections between the neurons that express these genes. Interestingly, *Drg11* and *Er81* are required for the formation of complementary somatosensory circuits: the for-

mer for the central projections of cutaneous afferents and the latter for those of muscle afferents. Therefore, in at least some cases "transcriptional matching" between pre- and post-synaptic neurons indeed reflects a role in neural circuit formation (Saito et al., 1995; Lin et al., 1998).

Despite their requirement for proper connectivity, however, there is no definitive evidence that ER81 and DRG11 are actually essential in both the pre- and post-synaptic neuronal populations that express them. The known functional requirement for ER81 localizes to sensory neurons (Arber et al., 2000), but the available data do not exclude a function in motoneurons as well. In the case of DRG11, the cellular locus of the connectivity defect is not yet clear. Conditional knockouts of *Drg11*, as well as identification of its target genes, should help to clarify the important issue of whether its expression in both peripheral and central neurons is indeed essential to its requirement for their proper connectivity.

Experimental Procedures

Generation of DRG-11 Knockout Mice

A mouse 129/SvEv genomic library was screened with a rat *Drg11* cDNA probe, and one genomic clone containing the paired homeodomain region was isolated. Sequencing and restriction mapping revealed that this clone contains multiple coding exons which span >10 kb. To construct a targeting vector, an IRES-Tau-lacZ-neo cassette (Mombaerts et al., 1996) was fused to the third coding exon which contains the putative DNA binding region. Electroporation, selection, and blastocyst injection of AB-1 ES cells were essentially as described (Ramirez-Solis et al., 1993). Germline transmission of the mutation was confirmed by both Southern blotting and PCR. Subsequent genotyping was done by PCR. Primers for Neo are 5′ GAT,CTC,CTG,TCA,TCT,CAC,CT 3′ and 5′ ATG,GGT,CAC,GAC, GAG,ATC,CT 3′, for the deleted region are 5′ TGC,AAA,GCA,AAT, CTG,ACC,GCT,CTG 3′ and 5′ GAA,CAG,AAA,CAG,CAT,GGA,GGA, AAC 3′.

We performed a series of RT-PCR experiments to characterize the nature of any transcripts produced from the mutated allele, using as a template cDNA prepared from wild-type and *Drg11*$^{-/-}$ spinal cord. We were unable to detect any transcripts using 5′ primers located in the *neo*^{r} gene and 3′ primers located in the *Drg11* coding region downstream of the deleted region. Nor were we able to detect any transcripts that encoded residual homeodomain sequences upstream of the deleted coding exons, or that spliced across these exons to downstream *Drg11* coding exons. However, we did detect a transcript using 5′ and 3′ primers located internal to the *lacZ* gene. The fact that no Xgal reaction product was detected suggests that this may reflect aberrant splicing that does not produce a functional *lacZ* transcript.

Behavioral Tests

Three different tests were used to assess thermal sensitivity (see Cao et al., [1998]). In the hot plate test, the animal was placed on a 52.5°C hot plate and the response latency to hind paw licking or to jumping was recorded. In the tail-flick test, the cut off time was 10 s to minimize damage to the tail. In the paw withdrawal test, the maximal stimulation time was 20 s (Hargreaves et al., 1988).

Mechanical sensitivity was assessed by a set of calibrated von Frey hairs using the up-down paradigm (Chaplan et al., 1994). The testing paradigm was initiated with the 0.3 g filament and the cut off in the absence of a response was 2.5 g (Malmberg et al., 1997).

Chemical nociception was assessed by the formalin and capsaicin tests (Dickenson and Sullivan, 1987; Puig and Sorkin, 1996). For the formalin test, the mice received a 15 μl intraplantar (i.pl.) injection of 5% formalin, and the licking behavior of the injected paw was observed over 40 min in 2 min periods at 5 min intervals. Licking behavior was also measured after i.pl. injection of capsaicin (2.5 μg/10 μl). In both the formalin and capsaicin tests, we determined the magnitude of the inflammatory response by measuring paw diameter with a spring-loaded caliper (Mitutoyo) at 40 or 30 min, respectively, after the formalin or capsaicin injection.

Motor function was assessed by using an accelarating rotarod treadmill (Ugo Basile, Comerio, Italy). The mice are first trained to walk on the rotating rod at a slow speed. After this, the mice undergo three trials in which the time spent on the accelerating rotating rod is determined. The mean of the three trials is considered representative for each animal. In all tests n = 7–8 animals of each genotype (+/+ or *Drg11*$^{-/-}$) were used. Analysis of *Drg11*$^{+/-}$ animals (n = 4) indicated no difference from wild-type in all of the behavioral tests performed.

Immunohistochemistry and In Situ Hybridization

Immunohistochemical staining and in situ hybridization were essentially done as described (Birren et al., 1993; Chen et al., 1998b; Ma et al., 1999). Antibodies used were: Mouse anti-NeuN (Chemicon, 1:500), rabbit anti-CGRP (Chemicon, 1:500), rabbit anti-calbindin D-28K (Chemicon, 1:1000), rabbit anti-peripherin (Chemicon, 1:500), rabbit anti-substance P (Peninsula, 1:500), rabbit anti-trkA (1:3000), rabbit anti-calretinin (Chemicon, 1:2000), rabbit anti-PKCγ (Santa Cruz, 1:500). Nissl staining was performed using 0.5% crest violet for 15 min. TUNEL labeling to detect apoptotic cells (Gavrieli et al., 1992) was performed using an ApopTag Peroxidase Kit (Intergen), according to the manufacturer's instructions. DiI tracing was performed as described (White and Behar, 2000). Cell counts were determined on Nissl-stained 7 μm plastic sections by stereological evaluation using a systematic random sampling procedure (Gundersen and Jensen, 1987).

Acknowledgments

We thank Shirley Pease and the staff of TAFCIT for production and maintenance of mutant mice, Gaby Mosconi for laboratory management, Heather Gilbert for technical help, Richard Behringer for AB-1 ES cells and STO cells, P. Mombaerts for the IRES-Tau-lacZ plasmid, Louis F. Reichardt and David Julius for antibodies, Marc Tessier-Lavigne for Slit and netrin probes, Haiming Xu for assistance with RT-PCR experiments, T. Saito for Figure 2, and Artur Kania, Tom Jessell, and Randy Johnson for sharing unpublished data on the *Lmx1b* knockout and for providing various probes and reagents. We are grateful to Tom Jessell for helpful comments on the manuscript. Animal experiments were reviewed and approved by the Animal Use and Care Committees (IACUCs) at Caltech and at UCSF. S.R. was supported in the laboratory of D.J.A. by a Gulbenkian Foundation Fellowship. Supported by NIH grant NS38253-01 to C.J.W. and NS 14627 to A.I.B. D.J.A. is an Investigator of the Howard Hughes Medical Institute.

Received October 24, 2000; revised April 18, 2001.

References

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S., and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene *atonal* is a positive transcriptional regulator expressed in the developing nervous system. J. Biol. Chem. *270*, 8730–8738.

Arber, S., Ladle, D.R., Lin, J.H., Frank, E., and Jessell, T.M. (2000). ETS gene *Er81* controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell *101*, 485–498.

Baba, H., Doubell, T.P., and Woolf, C.J. (1999). Peripheral inflammation facilitates Abeta fiber-mediated synaptic input to the substantia gelatinosa of the adult rat spinal cord. J. Neurosci. *19*, 859–867.

Bennett, D.L., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B., and Priestley, J.V. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J. Neurosci. *18*, 3059–3072.

Birren, S.J., Lo, L.C., and Anderson, D.J. (1993). Sympathetic neu-

rons undergo a developmental switch in trophic dependence. Development *119*, 597–610.

Brown, A.G., Rose, P.K., and Snow, P.J. (1977). The morphology of hair follicle afferent fibre collaterals in the spinal cord of the cat. J. Physiol. *272*, 779–797.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors—a molecular basis for odor recognition. Cell *65*, 175–187.

Cao, Y.Q., Mantyh, P.W., Carlson, E.J., Gillespie, A.M., Epstein, C.J., and Basbaum, A.I. (1998). Primary afferent tachykinins are required to experience moderate to intense pain. Nature *392*, 390–394.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., and Yaksh, T.L. (1994). Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods *53*, 55–63.

Chen, H., Bagri, A., Zupicich, J.A., Zou, Y., Stoeckli, E., Pleasure, S.J., Lowenstein, D.H., Skarnes, W.C., Chedotal, A., and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron *25*, 43–56.

Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K.C., Pepicelli, C.V., Gan, L., Lee, B., and Johnson, R.L. (1998a). Lmx1b: Involvement of Lmx1b in human nail patella syndrome. Nat. Genet. *19*, 51–55.

Chen, Z.-F., Paquette, A.J., and Anderson, D.J. (1998b). NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. Nat. Genet. *20*, 136–142.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., et al. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell *76*, 1001–1011.

Dickenson, A.H., and Sullivan, A.F. (1987). Peripheral origins and central modulation of subcutaneous formalin- induced activity of rat dorsal horn neurones. Neurosci. Lett. *83*, 207–211.

Emfors, P., Lee, K.-F., Kucera, J., and Jaenisch, R. (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell *77*, 503–512.

Escurat, M., Djabali, K., Gumpel, M., Gros, F., and Portier, M.M. (1990). Differential expression of two neuronal intermediate filament proteins, peripherin and the low-molecular-mass neurofilament protein (NF-L), during the development of the rat. J. Neurosci. *10*, 764–784.

Fariñas, I., Jones, K.R., Backus, C., Wang, X.-Y., and Reichardt, L.F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature *369*, 658–661.

Garel, S., Marin, F., Mattei, M.G., Vesque, C., Vincent, A., and Charnay, P. (1997). Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. Dev. Dyn. *210*, 191–205.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119*, 493–501.

Giger, R.J., Cloutier, J.F., Sahay, A., Prinjha, R.K., Levengood, D.V., Moore, S.E., Pickering, S., Simmons, D., Rastan, S., Walsh, F.S., et al. (2000). Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron *25*, 29–41.

Goldstein, M.E., House, S.B., and Gainer, H. (1991). NF-L and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. J. Neurosci. Res. *30*, 92–104.

Goulding, M., Lumsden, A., and Gruss, P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. Development *117*, 1001–1016.

Gundersen, H.J.G., and Jensen, E.B. (1987). The efficiency of systematic sampling in stereology and its prediction. J. Microscopy *147*, 229–263.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain *32*, 77–88.

He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a recep the axonal chemorepellent Semaphorin III. Cell *90*, 739–751.

Hogan, B., Costantini, F., and Lacy, E. (1986). Manipulati mouse embryo: A laboratory manual. (Cold Spring Harbor, N Spring Harbor Laboratory Press).

Honda, C.N. (1995). Differential distribution of calbindin-D2 parvalbumin in somatic and visceral sensory neurons. Neuros *68*, 883–892.

Huang, E.J., Zang, K., Schmidt, A., Saulys, A., Xiang, M., and ardt, L.F. (1999). POU domain factor Brn-3a controls the diffe tion and survival of trigeminal neurons by regulating Trk re expression. Development *126*, 2869–2882.

Hunt, S.P., Mantyh, P.W., and Priestly, J.V. (1992). The Organ of Biochemically Characterized Sensory Neurons. In Sensor rons: Diversity, Development, and Plasticity, S.A. Scott, ed York: Oxford University Press), pp. 60–76.

Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguc Bekku, Y., Yagi, T., and Fujisawa, H. (1997). Neuropilin-sema III/D-mediated chemorepulsive signals play a crucial role in eral nerve projection in mice. Neuron *19*, 995–1005.

Klein, R., Silos-Santiago, I., Smeyne, R.J., Lira, S.A., Brambi Bryant, S., Zhang, L., Snider, W.D. and Barbacid, M. (1994). tion of the neurotrophin-3 receptor gene trkC eliminates Ia afferents and results in abnormal movements. Nature *368*, 24

Knyihar-Csillik, E., Bezzegh, A., Boti, S., and Csillik, B. (1986) mine monophosphatase: a genuine marker for transganglioni lation of primary sensory neurons. J. Histochem. Cytoche 363–371.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Gige and Ginty, D.D. (1997). Neuropilin is a semaphorin III recepto *90*, 753–762.

Leonardo, E.D., Hinck, L., Masu, M., Keino-Masu, K., Faz Stoeckli, E.T., Ackerman, S.L., Weinberg, R.A., and Tessier-L M. (1997). Guidance of developing axons by netrin-1 and its tors. Cold Spring Harb. Symp. Quant. Biol. *62*, 467–478.

Lewin, G.R. (1996). Neurotrophins and the specification of ne phenotype. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *351*, 40

Liem, K.F., Jr., Tremml, G., and Jessell, T.M. (1997). A r the roof plate and its resident TGFβ-related proteins in ne patterning in the dorsal spinal cord. Cell *91*, 127–138.

Lin, J.H., Saito, T., Anderson, D.J., Lance-Jones, C., Jessel and Arber, S. (1998). Functionally-related motor neuron po muscle sensory afferent subtypes defined by coordinate *ET* expression. Cell *95*, 393–407.

Logan, C., Wingate, R.J.T., McKay, I.J., and Lumsden, A. *Tlx-1* and *Tlx-3* homeobox gene expression in cranial senso glia and hindbrain of the chick embryo: markers of patterne nectivity. J. Neurosci. *18*, 5389–5402.

Ma, Q., Fode, C., Guillemot, F., and Anderson, D.J. NEUROGENIN1 and NEUROGENIN2 control two distinct wa neurogenesis in developing dorsal root ganglia. Genes & D 1717–1728.

Malmberg, A.B., Chen, C., Tonegawa, S., and Basbaum, A.I. Preserved acute pain and reduced neuropathic pain in mice PKCgamma. Science *278*, 279–283.

McEvilly, R.J., Erkman, L., Luo, L., Sawchenko, P.E., Ryan, A. Rosenfeld, M.G. (1996). Requirement for Brn-3.0 in differe and survival of sensory and motor-neurons. Nature *384*, 57

Messersmith, E.K., Leonardo, E.D., Shatz, C.J., Tessier-Lavig Goodman, C.S., and Kolodkin, A.L. (1995). Semaphorin III ca tion as a selective chemorepellent to pattern sensory project the spinal cord. Neuron *14*, 949–959.

Mirnics, K., and Koerber, H.R. (1995). Prenatal development primary afferent fibers: II. Central projections. J. Comp. Neur 601–614.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., K., Wen, D., Yan, Q., and Snider, W.D. (1997). IB4-bindin neurons switch from NGF to GDNF dependence in early po life. Neuron *19*, 849–861.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Morin, X., Cremer, H., Hirsch, M.-R., Kapur, R.P., Goridis, C., and Brunet, J.-F. (1997). Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene *Phox2a*. Neuron *18*, 411–423.

Mullen, R.J., Buck, C.R., and Smith, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development *116*, 201–211.

Ozaki, S., and Snider, W.D. (1997). Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J. Comp. Neurol. *380*, 215–229.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J.-F. (1999). The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. Nature *399*, 366–370.

Puig, S., and Sorkin, L.S. (1996). Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. Pain *64*, 345–355.

Puschel, A.W., Adams, R.H., and Betz, H. (1996). The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. Mol. Cell. Neurosci. *7*, 419–431.

Ralston, H.J., Light, A.R., Ralston, D.D., and Perl, E.R. (1984). Morphology and synaptic relationships of physiologically identified low-threshold dorsal root axons stained with intra-axonal horseradish peroxidase in the cat and monkey. J. Neurophysiol. *51*, 777–792.

Ramirez-Solis, R., Davis, A.C., and Bradley, A. (1993). Gene targeting in embryonic stem cells. Methods Enzymol. *225*, 855–878.

Ren, K., Ruda, M.A., and Jacobowitz, D.M. (1993). Immunohistochemical localization of calretinin in the dorsal root ganglion and spinal cord of the rat. Brain Res. Bull. *31*, 13–22.

Saito, T., Greenwood, A., Sun, Q., and Anderson, D.J. (1995). Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol. Cell. Neurosci. *6*, 280–292.

Scott, S.A. (1992). Sensory neurons: Diversity, development and plasticity (Oxford: Oxford University Press).

Shortland, P., Woolf, C.J., and Fitzgerald, M. (1989). Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. J. Comp. Neurol. *289*, 416–433.

Silos-Santiago, I., Jeng, B., and Snider, W.D. (1995). Sensory afferents show appropriate somatotopy at the earliest stage of projection to dorsal horn. Neuroreport *6*, 861–865.

Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature *368*, 246–249.

Snider, W.D., and McMahon, S.B. (1998). Tackling pain at the source: new ideas about nociceptors. Neuron *20*, 629–632.

Snider, W.D., and Silos-Santiago, I. (1996). Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *351*, 395–403.

Snider, W.D., and Wright, D.E. (1996). Neurotrophins cause a new sensaton. Neuron *16*, 229–232.

Thomas, D.A., Dubner, R., and Ruda, M.A. (1994). Neonatal capsaicin treatment in rats results in scratching behavior with skin damage: potential model of non-painful dysesthesia. Neurosci. Lett. *171*, 101–104.

Tiveron, M.-C., Hirsch, M.-R., and Brunet, J.-F. (1996). The expression pattern of the transcription factor Phox2a delineates synaptic pathways of the autonomic nervous system. J. Neurosci. *16*, 7649–7660.

Wang, F., Nemes, A., Mendelsohn, M., and Axel, R. (1998). Odorant receptors govern the formation of a precise topographic map. Cell *93*, 47–60.

Wang, K.H., Brose, K., Arnott, D., Kidd, T., Goodman, C.S., Henzel, W., and Tessier-Lavigne, M. (1999). Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. Cell *96*, 771–784.

Wang, S.S., Tsai, R.Y.L., and Reed, R.L. (1997). The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. J. Neurosci. *17*, 4149–4158.

White, F.A., and Behar, O. (2000). The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. Dev. Biol. *225*, 79–86.

Willis, W.D., and Coggeshall, R.E. (1991). Sensory mechanisms of the spinal cord (New York: Plenum Press).

Wilson, P., and Kitchener, P.D. (1996). Plasticity of cutaneous primary afferent projections to the spinal dorsal horn. Prog. Neurobiol. *48*, 105–129.

Xiang, M.Q., Gan, L., Zhou, L., Klein, W.H., and Nathans, J. (1996). Targeted deletion of the mouse POU domain gene Brn-3a causes a selective loss of neurons in the brain-stem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. Proc. Natl. Acad. Sci. USA *93*, 11950–11955.

Xu, Y., Baldassare, M., Fisher, P., Rathbun, G., Oltz, E.M., Yancopoulos, G.D., Jessell, T.M., and Alt, F.W. (1993). LH-2: A LIM/homeodomain gene expressed in developing lymphocytes and neural cells. Proc. Natl. Acad. Sci. USA *90*, 227–231.

Yoshimura, M., and Jessell, T.M. (1989). Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons in vitro. J. Neurophysiol. *62*, 96–108.

Yoshimura, M., and Nishi, S. (1993). Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. Neuroscience *53*, 519–526.

Yoshimura, M., and Nishi, S. (1995). Primary afferent-evoked glycine- and GABA-mediated IPSPs in substantia gelatinosa neurones in the rat spinal cord in vitro. J. Physiol. (Lond.) *482*, 29–38.

ELSEVIER

MCN Molecular and Cellular Neuroscience

www.elsevier.com/locate/ymcne
Mol. Cell. Neurosci. 33 (2006) 236–246

Involvement of DRG11 in the development of the primary afferent nociceptive system

Sandra Rebelo,[a] Zhou-Feng Chen,[b] David J. Anderson,[c] and Deolinda Lima[a,*]

[a]*Laboratory of Molecular Cell Biology, Faculty of Medicine of Oporto and IBMC, University of Oporto, Porto, Portugal*
[b]*Departments of Anesthesiology, Psychiatry, Molecular Biology and Pharmacology, Washington University School of Medicine Pain Center, St. Louis, MO 63110, USA*
[c]*Division of Biology 216-76 and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125, USA*

Received 20 March 2006; revised 14 July 2006; accepted 26 July 2006
Available online 15 September 2006

During development, dorsal root ganglia (DRG) neurons differentiate in various subpopulations, nociceptive neurons belonging in the small-diameter class. This study addresses the role played by DRG11, a transcription factor expressed in the spinal area of projection of small-diameter DRG neurons, in the development of the primary afferent system. The various subclasses of DRG neurons were compared between wild-type and *Drg11*$^{-/-}$ mice at embryonic and postnatal life. In *Drg11*$^{-/-}$ mice, numbers of small peptidergic and non-peptidergic DRG neurons were decreased at P7 concomitant with abnormal cell death. Innervation by small DRG neurons was impaired in cutaneous, visceral and deep tissues. Large DRG neurons were not affected. The data point to a role for DRG11 in early postnatal survival of normally generated small primary afferent neurons innervating various kinds of peripheral tissues, which would explain the nociceptive deficits observed in *Drg11*-null mutant mice.

Introduction

Dorsal root ganglion (DRG) cells can be classified in various subpopulations based on their anatomy, physiology and neurochemistry (Lawson, 1992; Snider and McMahon, 1998; for review, see Hunt and Mantyh, 2001). The population of large-diameter sensory neurons has myelinated axons and conveys input from proprioceptive and mechanoceptive endings in the skin. Neurons with myelinated axons can be distinguished by their content on phosphorylated heavy-chain neurofilament (NF200) and represent approximately 40% of lumbar DRG cells (Lawson and Waddell, 1991). The population of small-diameter sensory neurons has either unmyelinated (C) or thinly myelinated (Aδ) axons and receives nociceptive input and innocuous thermal input from a variety of peripheral tissues including skin, viscera and deep tissues (muscles and joints) (McCarthy and Lawson, 1990; Fundin et al., 1997). Small sensory neurons can be further subdivided in peptidergic and non-peptidergic. Peptidergic neurons (about 40% of the total DRG cells) express neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP), as well as the nerve growth factor tyrosine kinase receptor A (trkA) (Averill et al., 1995; Michael et al., 1997). Non-peptidergic neurons (about 30% of the total DRG cells) do not express neuropeptides, but exhibit fluoride-resistant acid phosphatase (FRAP) and thiamine monophosphatase (TMP) activity (Silvermann and Kruger, 1990). They bind to the lectin *Griffonia simplicifolia* (IB4) and are recognized by the monoclonal antibody LA4 (Nagy and Hunt, 1982; Streit et al., 1986; Alvarez et al., 1991). These two subpopulations of small-diameter sensory neurons differ in their central projections. Peptidergic neurons terminate in lamina I and outer lamina II while non-peptidergic neurons project to inner lamina II (Coimbra et al., 1974; Wang et al., 1994; Molliver et al., 1995). Recently, it has been shown that these neurons differ in their peripheral projections to the epidermis, as well (Zylka et al., 2005). Peptidergic neurons terminate in the stratum spinosum, while non-peptidergic Mrgprd$^+$ neurons terminate in the stratum granulosum. They are also distinct as to their requirements for neurotrophic factors (for review, see Snider and McMahon, 1998): While peptidergic neurons continue to depend on nerve growth factor (NGF) for growth and survival after birth (Silos-Santiago et al., 1995; Molliver and Snider, 1997), non-peptidergic (IB4-positive) neurons down-regulate trkA at perinatal ages (Bennett et al., 1996a) and begin to express the glial-derived neurotrophic factor (GDNF) receptor complex, including the GFRα and c-ret subunits, and to respond to GDNF (Molliver et al., 1997; Bennett et al., 1998, 2000; Orozco et al., 2001; Zwick et al., 2002).

Despite such diversity, data on the factors that determine the anatomical and functional differentiation of sensory neurons are still scarce. It is known that DRG cells are born in successive waves, beginning by large cells and followed by the more

* Corresponding author. Instituto de Histologia e Embriologia, Faculdade de Medicina do Porto, Alameda Hernâni Monteiro, 4200-319 Porto, Portugal. Fax: +1 351 225513655.
E-mail address: limad@med.up.pt (D. Lima).
Available online on ScienceDirect (www.sciencedirect.com).

1044-7431/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2006.07.013

numerous small cells. In the mouse, large-diameter neurons are born between E9.5 and E11.5 and small-diameter neurons between E10.5 and E13.5 (Lawson and Biscoe, 1979; Fariñas et al., 2002). A later wave of nociceptive neurons arising from the neural crest-derived boundary cap region enters the DRG after E11.5 (Maro et al., 2004). In addition, a subpopulation of small-diameter neurons, probably the one that expresses CGRP, is produced at E15.5–16.5 in the rat (Kitao et al., 1996). DRG sensory neurons appear to initiate axon elongation immediately after they are generated. Although sensory projections begin to exit the ganglia at E10, most of them only meet their final targets at the periphery and spinal cord late in embryogenesis (Reynolds et al., 1991; Fariñas et al., 1996; White et al., 1996).

Transcription factors play a fundamental role in embryonic development. They are expressed sequentially in a region- and cell-specific manner (Jessell, 2000) and act by defining progenitor domains, restricting developmental potentials and establishing diverse differentiation from each domain (Jessell, 2000; Zhuang and Sockanathan, 2006). DRG11 is a paired homeodomain protein described by Saito et al. (1995), which is expressed in primary afferent sensory neurons and their target areas in the spinal cord superficial dorsal horn. The involvement of the superficial dorsal horn in nociceptive processing suggested that DRG11 might be relevant for the development of the nociceptive system, in particular the differentiation of nociceptors and the establishment of their first relay in the spinal cord. By studying the phenotype of *Drg11*$^{-/-}$ adult mice, it was later shown that this gene is indeed essential for the acquisition of normal pain performance in several nociceptive tests (Chen et al., 2001). Mutant animals exhibited a substantial reduction in the sensitivity to a broad range of noxious stimuli, while sensorimotor function was apparently normal (Chen et al., 2001). Structural defects occurred both at the DRG and the spinal cord. The DRG contained reduced numbers of trkA$^+$ and CGRP$^+$ neurons at adulthood. The spinal cord dorsal horn appeared distorted in Nissl-stained specimens, which may be due to aberrant migration during embryonic development (Ding et al., 2004). Furthermore, the dorsal horn lacked the expression of PKCγ in the entire lamina II and presented scarce and medially shifted substance P and CGRP primary afferent projections (Chen et al., 2001).

Interestingly, in *Drg11* knockout mice, primary afferent fibers approached the spinal cord forming what looked like a normal dorsal root at the appropriate time point of development. However, they stopped at the dorsal root entry zone for 2 days before they penetrated the spinal gray matter (Chen et al., 2001). Based on this finding, we hypothesized that DRG11 is not involved in primary afferent neuronal differentiation, but rather in their target recognition and/or survival. The present study further investigates this question by determining the time at which reductions in the number of sensory neurons in various categories first become apparent.

A time course analysis was performed, from embryonic age E14.5 to adulthood, of the numbers of large- and small-diameter peptidergic and non-peptidergic DRG neurons in *Drg11*$^{-/-}$ mice, as compared to wild-type mice. A parallel analysis was also carried out of the extent to which innervation of various peripheral tissues was affected. In *Drg11*$^{-/-}$ mice, the numbers of both small and large size DRG neurons were normal until perinatal life. A marked decrease in both CGRP- and IB4-positive small-diameter primary afferent neurons, but not in large-diameter primary afferent neurons, was first observed at P7. This decrease affected the innervation of cutaneous, visceral and deep peripheral tissues, visceral innervation being more markedly reduced. These data indicate that *Drg11* is required for the survival of small-diameter peptidergic and non-peptidergic neurons that innervate various peripheral tissues, but not for their initial differentiation.

This work has been partly presented as a preliminary report in abstract form (Rebelo et al., 2001).

Results

DRG neurons affected by Drg11 deletion

In our previous study aimed at the functional characterization of *Drg11*$^{-/-}$ adult mice, the decrease of responsiveness in nociceptive behavioral tests was accompanied by a reduction in the amount of trkA- and CGRP-positive DRG neurons (Chen et al., 2001). These data suggested that a deficit in nociceptive innervation was at the basis of reduced pain perception. It was not clear, however, whether non-peptidergic small size DRG neurons were compromised nor was it determined whether large size primary sensory neurons, mediating innocuous sensory processing, were also affected. In order to address this question, we now quantified, by the use of stereological approaches, the total amount of neurons (Nissl stained), and the amount of large size (NF200-immunoreactive), small size peptidergic (CGRP-immunoreactive) and small size non-peptidergic (stained for the lectin IB4) neurons in the L3 and L4 DRGs of wild-type and *Drg11*$^{-/-}$ adult mice (P21–30). The frequency distribution of cell body diameters of neurons belonging in each cell group was determined.

In *Drg11*$^{-/-}$ mice, total number of DRG neurons was decreased to approximately 2/3 of the number present in wild-type mice. A marked reduction was observed in the number of CGRP$^+$ and IB4$^+$ neurons, but not in the number of NF200$^+$ neurons (Table 1). The cell size distribution histogram of the whole DRG neuronal population was shifted to the right in knockout compared to wild-type mice due to the decrease of the proportion of both C (250–500 μm^2) and Aδ (500–750 μm^2) neurons and the consequent increase of the proportion of large neurons (Fig. 1A). The size distribution of neurons stained for NF200 was similar in wild-type and knockout mice (Fig. 1B), in accordance with the lack of effect upon large-diameter DRG neurons (Table 1). In the case of CGRP-immunoreactive cells, the decrease observed in knockout mice (see Table 1) affected only neurons within the C size range (250–500 μm^2) (Fig. 1C), indicating that the *Drg11*-dependent peptidergic primary afferents belong mainly in the C neuronal group. As to IB4-positive cells, no differences were observed in the cell size distribution histogram between wild-type and *Drg11*$^{-/-}$ mice (Fig. 1D), indicating that the numerical reduction shown in Table 1 crosses the entire population of non-peptidergic neurons.

Table 1
Number of sensory neurons in lumbar DRG of adult wild-type and *Drg11*$^{-/-}$ mice

Genotype	Nissl	NF200	CGRP	IB4
+/+	9915±1422	5631±390	3038±77	2977±641
Drg11$^{-/-}$	7073±1813	5112±544	1979±116	1786±697
% reduction	29	–	35	40
	$p<0.05$	NS	$p<1.6\times10^{-5}$	$p<0.04$

Values represent the average ± SEM of the total number of neurons present in the L3 or L4 DRGs. NS—not significantly different.

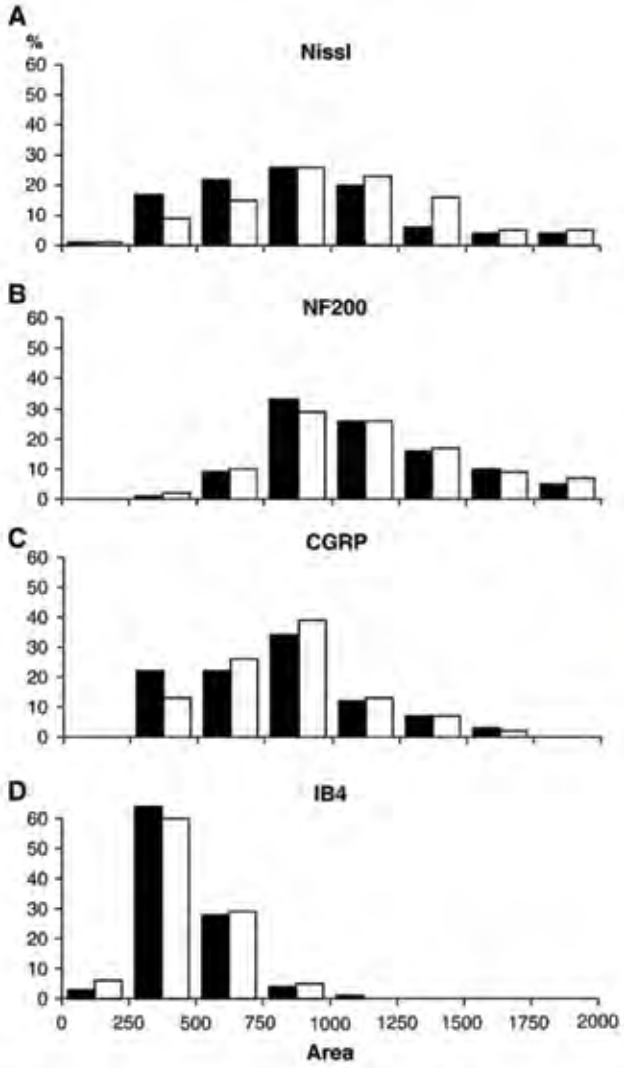

Fig. 1. Frequency distribution of the cell body areas of DRG neurons in adult wild-type and *Drg11*$^{+/+}$ mice. Cross-section cell body areas of DRG neurons of wild-type (full bars) and *Drg11*$^{-/-}$ (open bars) mice, at a total of 100 neurons per animal group, were distributed at 250 μm^2 intervals. (A) The distribution concerning the whole DRG population, stained by the Nissl method, was shifted to the right in *Drg11*$^{-/-}$ mice as compared to wild-type due to a decrease of the relative amount of neurons measuring 250 to 750 μm^2 and an increase of the relative amount of neurons measuring more than 1000 μm^2, which agrees with the underrepresentation of small but not large neurons in *Drg11* knockout mice shown in Table 1. (B) The distribution of large-diameter cells, stained for NF200, was similar in both wild-type and *Drg11*$^{-/-}$ mice, as expected from Table 1. (C) The distribution of small CGRP-immunoreactive neurons was shifted to the right in *Drg11*$^{-/-}$ mice due to a decrease in the population measuring 250 to 500 μm^2 suggesting that *DRG11* deletion affects mainly a subpopulation of CGRP neurons comprised in the smaller size range. (D) The distribution of IB4-positive neurons was not changed in *Drg11*$^{-/-}$ mice as compared to wild-type mice, suggesting that the decrease in number of IB4 neurons shown in Table 1 affects the entire size range.

These data show that the primary afferent neuronal population controlled by DRG11 is restricted to a fraction of both peptidergic and non-peptidergic small neurons with sizes ranging from 250 to 750 μm^2. Moreover, DRG11 appears to affect non-peptidergic neurons of all sizes, but mainly the smaller fraction (C class) of CGRP neurons. These findings support the specific involvement of DRG11 in the development of the nociceptive system.

Effect of Drg11 deletion upon DRG neuronal differentiation

The underrepresentation of both classes of small size primary afferent neurons in *Drg11*$^{-/-}$ mice at adulthood could either reflect abnormal formation from the beginning of neuronal differentiation or disturbance in the differentiation process during embryonic development. Alternatively, neurons could differentiate normally but be unable to survive at a certain time point. To clarify this issue, the way in which each neuronal population evolved in number during development was evaluated in wild-type and *Drg11*$^{-/-}$ mice. Stereological counting of Nissl stained neurons, neurons immunoreactive for NF200, neurons immunoreactive for trkA (embryonic life) or CGRP (from P0 on) and neurons stained for IB4 (from P0 on) was performed in wild-type and *Drg11*$^{-/-}$ mice at ages E14.5, E18.5, P0, P7, P14 and P21–30 (adulthood). (Staining for CGRP and IB4 was observed only from P0 since it is around birth that non-peptidergic neurons lose NGF dependence, down-regulate trkA expression and become responsive to IB4 (Bennett et al., 1996a, 1998; Molliver et al., 1997). Therefore, counts of trkA-positive neurons at embryonic stages include both presumptive peptidergic and non-peptidergic primary afferent neurons.)

In both wild-type and knockout mice, total numbers of DRG neurons were reduced from E14.5 to E18.5, although statistically significant differences could only be detected in knockout mice from E14.5 to E18.5 (Fig. 2). From E18.5 to P0, numbers slightly increased. Postnatally, total numbers kept at similar values until adulthood, but were significantly smaller in knockout mice compared to wild-type mice at P14 and adulthood (Fig. 2). Numbers of large size neurons did not vary significantly along embryonic development. Postnatally, however, they increased progressively although not reaching statistical significance (Fig. 2). No differences were observed between wild-type and knockout mice at any time point. Small size neurons increased in numbers in both wild-type and knockout mice from E14.5 until birth, the difference being particularly marked at P0 if added numbers of CGRP$^+$ and IB4$^+$ neurons are compared with those of trkA at previous time points (Fig. 2). Postnatally, in wild-type animals, numbers increased until P14 and then decreased slightly, although statistic significance for CGRP$^+$ neurons was only achieved between P7 and P14 (Fig. 2). In knockout animals, such variations were not so clear, save for CGRP$^+$ neurons between P7 and P14. The amount of trkA$^+$ neurons in knockout mice was identical to that in wild-type mice at E14.5 and E18.5. No differences were detected also for CGRP$^+$ and IB4$^+$ neurons at P0 (Fig. 2). From P7 until adulthood, the number of peptidergic and non-peptidergic small size neurons in knockout mice was significantly smaller than that occurring in wild-type mice.

According to these data, DRG11 has no effect on the development of large size primary afferent neurons. The development of small size peptidergic and non-peptidergic neurons appears to be not affected until birth. After birth, their numbers become significantly smaller in Drg11$^{-/-}$ mice as compared to wild-type animals, although approximately half of the neurons of each class are preserved until adulthood. DRG11 thus seems to be required for the maintenance of a significant fraction of both peptidergic and non-peptidergic classes of small primary afferent neurons immediately after birth.

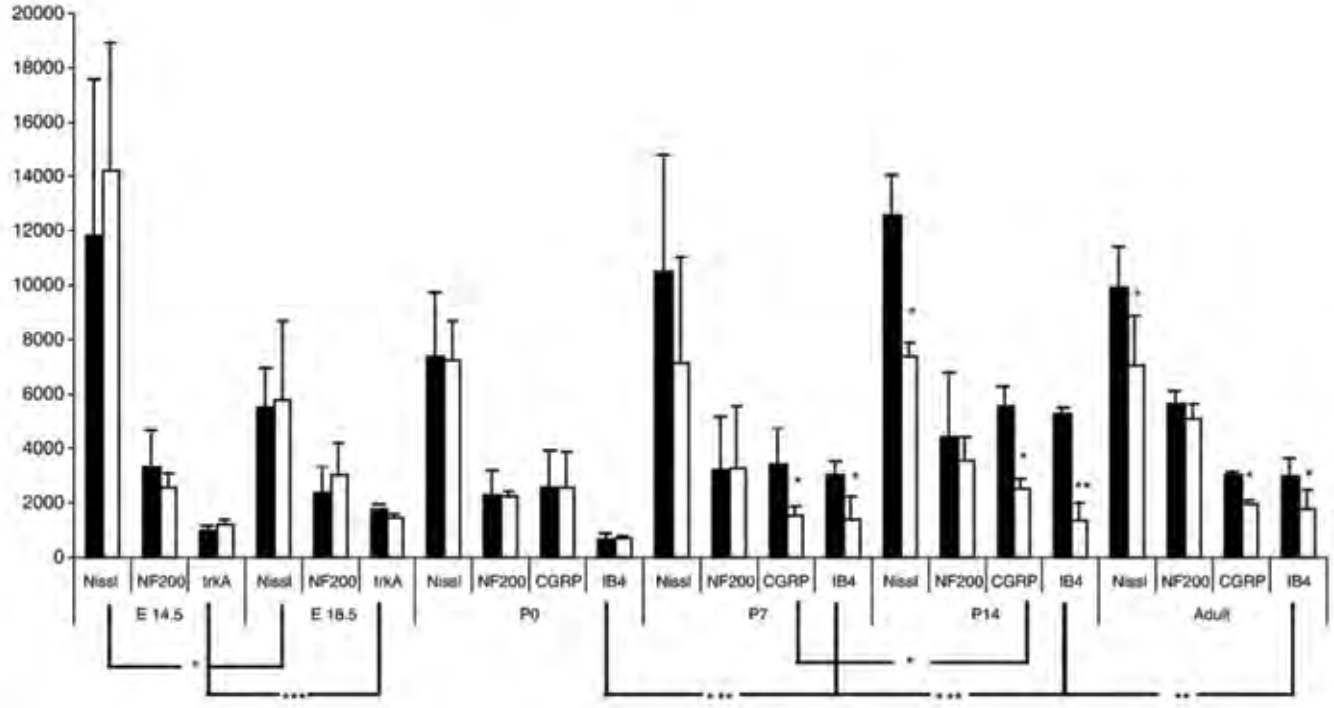

Fig. 2. Numbers of lumbar DRG neurons in wild-type and *Drg11*$^{-/-}$ mice along development. Numbers of neurons stained by the Nissl method (whole DRG population), for NF200 (large DRG population), trkA (small DRG population during embryonic development), CGRP (postnatal small peptidergic DRG population) and for IB4 (postnatal small non-peptidergic DRG population) in wild-type (full bars) and *Drg11*$^{-/-}$ (open bars) mice at ages E14.5, E18.5, P0, P7, P14 and adulthood. Both CGRP and IB4 neurons are underrepresented in *Drg11*$^{-/-}$ mice as compared to wild-type mice from P7 on. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Apoptosis in the DRG of Drg11$^{-/-}$ *mice*

The data collected up to this point suggested that the decreased numbers of both peptidergic and non-peptidergic small size neurons observed at P7 might be due to either lack of further differentiation, such as impairment of phenotypic changes occurring after birth (in particular expression of IB4) or to neuronal degeneration. In order to clarify this point, we investigated whether abnormal apoptosis takes place postnatally and quantified pyknotic neuronal profiles in the DRG of wild-type and mutant animals at P7. Immunodetection of the active form of caspase-3, a ubiquitously distributed caspase that is a main effector of the apoptotic cascade (for review, see Yuan and Yankner, 2000), was performed.

Punctate cytoplasmatic immunoreaction was observed in cells with nuclear and surface apoptotic morphology, but not in cells showing normal morphology, which is in accordance with data from Coggeshall et al. (1994). At P0, only seldom caspase-3-immunostained profiles were seen in wild-type mice (Fig. 3A) and *Drg11*$^{-/-}$ (Fig. 3B), whereas at P7 the amount of apoptotic cells was much lower in wild-type (4.0 ± 1.9; $p<0.003$) (Fig. 3C) than in *Drg11*$^{-/-}$ mice (39.6 ± 5.5) (Fig. 3D). In order to verify whether cells undergoing apoptosis belonged in the small-diameter subset of DRG neurons, we performed double immunolabeling for caspase-3 and the small primary afferent neuron marker peripherin in wild-type and *Drg11*$^{-/-}$ mice at the age of P7. In wild-type mice, immunocolocalization of caspase-3 and peripherin was seldom observed despite the large numbers of peripherin-positive cells (Fig. 3E). In *Drg11*$^{-/-}$ mice, all caspase-positive cells were also peripherin-positive (Fig. 3F, arrowheads). These results show that the DRG cells affected by Drg11 deletion are small primary afferent neurons, as suggested by the decrease in the number of CGRP- and IB4-positive cells in *Drg11*$^{-/-}$ mice. These observations indicate that the decrease in small, putative nociceptive peptidergic and non-peptidergic primary afferent neurons in Drg11$^{-/-}$ mice is at least partly due to programmed cell death taking place shortly after birth. The possibility that disturbances in the establishment of synaptic connectivity at the spinal cord dorsal horn account for small DRG neurons cell death needs to be evaluated in future studies and will be discussed in the light of our previous findings.

Peripheral target innervation in the absence of DRG11

Neuronal survival requires trophic support, which is dependent on the establishment of correct connections with the targets (Snider and Silos-Santiago, 1996; Kirstein and Fariñas, 2002; Markus et al., 2002). The apoptotic process resulting in the decrease in number of small DRG neurons could thus be due to failing of primary afferent axons in innervating their peripheral targets in the absence of DRG11. In spite of data from the rat showing that primary afferent neurons establish functional contacts with peripheral targets by E18–19 (Fitzgerald and Fulton, 1992; Hall et al., 1997; Jackman and Fitzgerald, 2000, for review, see Fitzgerald, 2005) and our observation that small DRG neurons are present in normal numbers in *Drg11*$^{-/-}$ mice at P0, we decided to evaluate this possibility. By doing so, we would also clarify whether peptidergic and non-peptidergic neurons spared in the

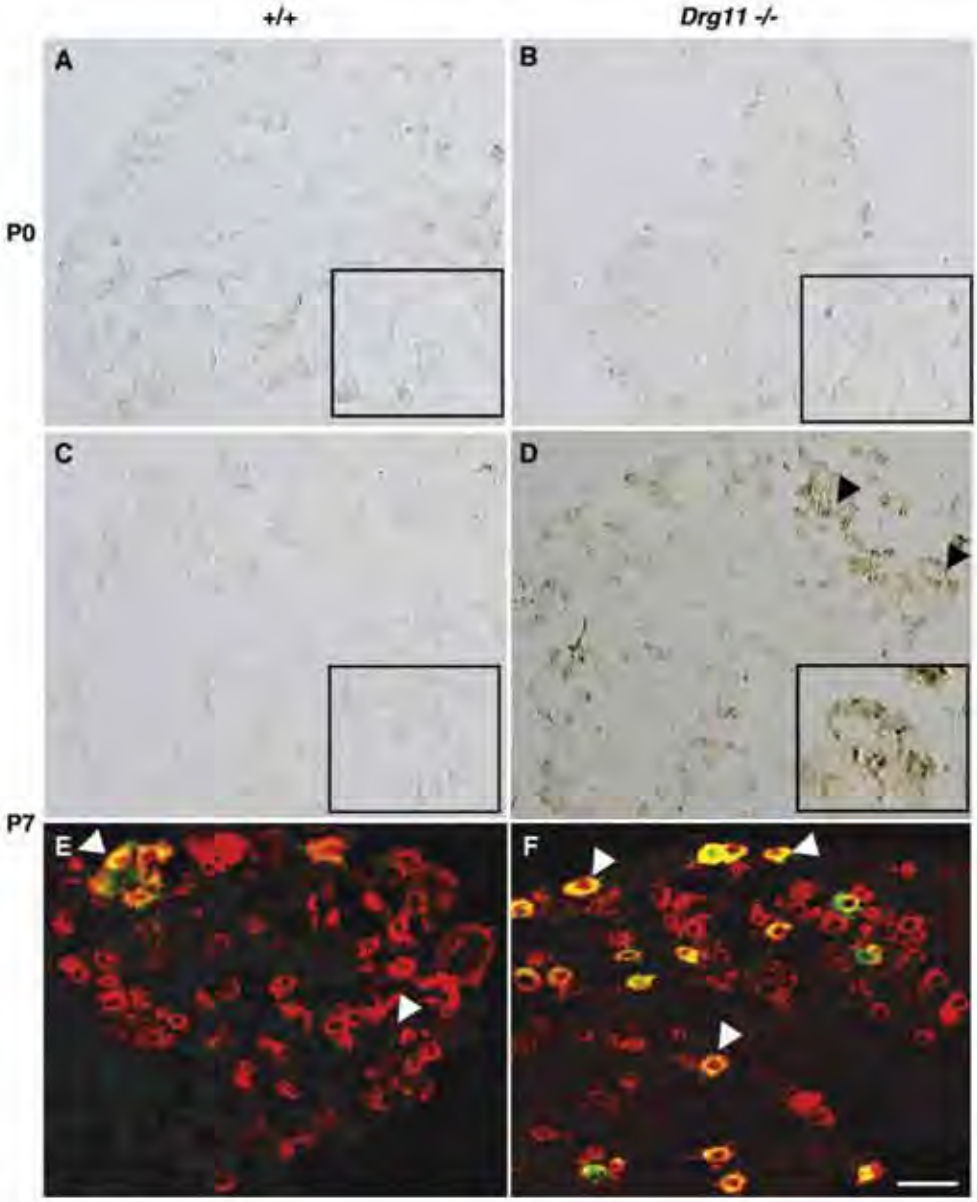

Fig. 3. Apoptosis in the DRG of *DRG11*$^{+/+}$ mice. Caspase-3 expression in the L4 DRG of wild-type (A, C, E) and *Drg11*$^{-/-}$ (B, D, F) mice at postnatal ages P0 (A, B) and P7 (C–F). Caspase-3 immunoreaction is detected at P7 in *Drg11*$^{-/-}$ mice (D). Simultaneous immunodetection of caspase-3 (green) and peripherin (red) (E, F) revealed numerous caspase-3 and peripherin double-positive cells in *Drg11*$^{-/-}$ mice (F; arrowheads), but only a few in wild-type mice (E; arrowhead). Scale bar—50 μm.

absence of DRG11 represent a subset of neurons innervating a particular peripheral tissue. Peripheral staining of both peptidergic and non-peptidergic primary afferents was performed from P0 on. CGRP-immunoreactive and IB4-positive fibers were quantified in cutaneous (lumbar and plantar skin), visceral (urinary bladder) and deep (knee joint sinovial sheet) tissue samples of wild-type and *Drg11*$^{-/-}$ mice at P0, P7, P14 and adulthood (Fig. 4).

The amount of CGRP^{+} and IB4^{+} fibers innervating both lumbar and plantar skin areas did not differ between wild-type and *Drg11*$^{-/-}$ mice at P0 (Figs. 4A, B). In wild-type mice, innervation by both groups of fibers increased until P7 and kept around that value from then on (Figs. 4A, B). In *Drg11*$^{-/-}$ mice, no increase in stained fibers occurred from P0 to P7, so that much fewer fibers of both types were stained at P7, P14 and adulthood (Figs. 4A, B and 5A, B). Innervation was nearly abolished in the epidermis (Fig. 5B) and considerably reduced in the dermis and sweat glands.

In the urinary bladder of adult wild-type mice, CGRP-immunoreactive fibers were stained abundantly in the muscular layer (Figs. 4C and 5C) and in fewer amounts in the mucosa (Fig. 4D). Clear staining of non-peptidergic fibers could not, however, be observed due to intense non-specific IB4 binding. A similar constraint was referred to previously in the rat (Avelino et al., 2002). According to results from Bennett et al. (1996b) showing that IB4 binding is higher in cutaneous than in visceral primary afferents, it is possible that IB4^{+} fibers contribute little to visceral innervation. A recent study on innervation by Mrgprd^{+} DRG neurons, which make up 75% of the IB4^{+} population, also failed to detect staining in the bladder (Zylka et al., 2005). In wild-type mice, CGRP-

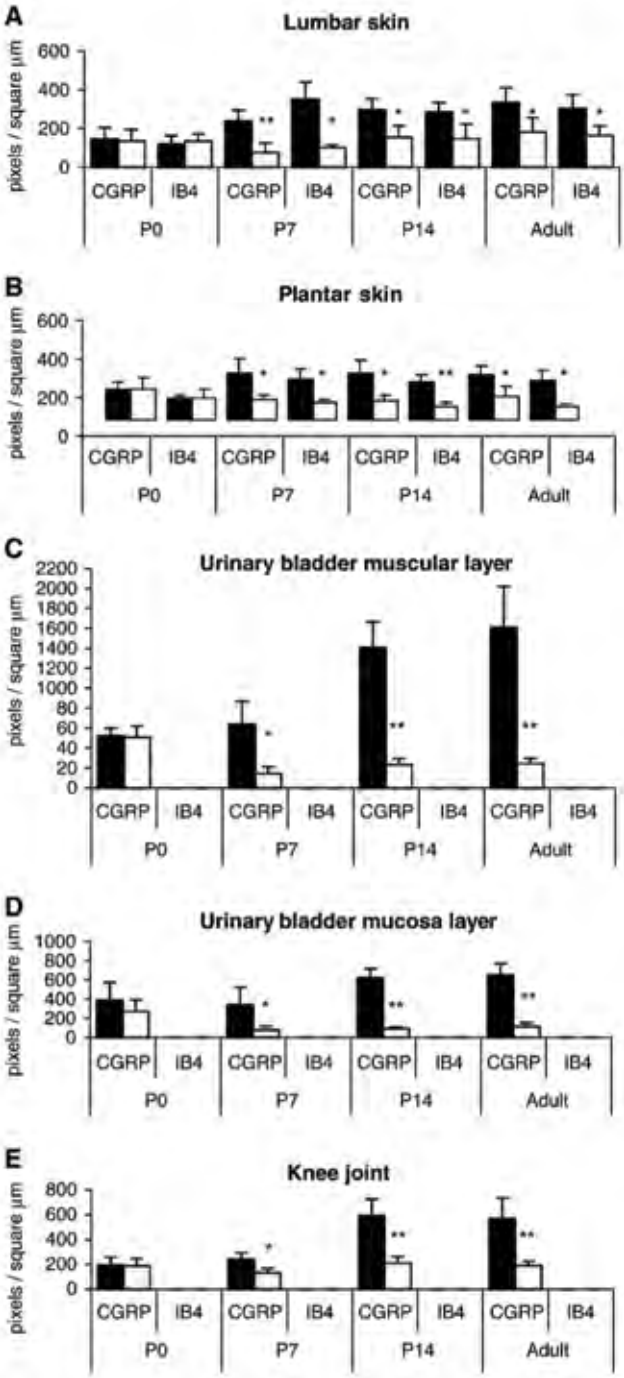

Fig. 4. Amount of peripheral innervation by small DRG neurons in wild-type and *Drg11*$^{-/-}$ mice. The amount of pixels per µm^2 occupied by CGRP- and IB4-positive fibers innervating the skin (A, B), urinary bladder (C, D) and knee sinovial sheet (E) at P0, P7, P14 and adulthood in wild-type (full bars) and *Drg11*$^{-/-}$ (open bars) mice is represented. Innervation was markedly decreased in all tissues in *Drg11*$^{-/-}$ mice as compared to the wild-type from P7 on. Note that IB4-positive fibers could only be detected in cutaneous tissue samples.

immunoreactive fibers increased in number from P0 up to P14 (Figs. 4C, D). In *Drg11*$^{-/-}$ mice, CGRP staining was similar to that of wild-type mice at P0, but decreased markedly from P0 to P7 and kept in such low amounts until adulthood (Figs. 4C, D and 5D).

Abundant CGRP-immunoreactive fibers were observed in the sinovial sheet of the knee joint of adult wild-type mice (Figs. 4E and 5E), as expected from other studies (Hanesch et al., 1991; Heppelmann et al., 1997; Ebinger et al., 2001). IB4 staining could not be observed, which is in accordance with previous data showing a complete absence of IB4-binding neurons labeled retrogradely from the rat knee joint (Ivanavicius et al., 2004) and very low numbers of IB4 neurons innervating other deep somatic structures (Ambalavanar et al., 2003; Bennett et al., 1996b). In wild-type mice, CGRP$^+$ fibers increased in number from P7 to P14 and kept around that value from then on (Fig. 4E). No differences were detected between wild-type and knockout mice at P0. At P7, P14 and adulthood, fewer fibers were observed in knockout mice as compared to wild-type mice (Figs. 4E and 5E, F).

The lack of differences between wild-type and knockout mice at P0 indicates that peripheral innervation can reach normal development at this time point in the absence of DRG11. However, since peripheral innervation continues to expand until later in postnatal life, the possibility that DRG11-dependent failure in target recognition or collateral sprouting after birth accounts for postnatal cell death cannot be ruled out. On the other hand, innervation was disrupted from P7 on in the various kinds of peripheral tissues studied, which does not support the possibility that the population of DRG11-dependent primary afferent neurons is tissue specific. Nevertheless, CGRP innervation appeared to be more severely compromised in the urinary bladder.

Discussion

Drg11 is required for the projection of sensory afferent fibers to the dorsal horn of the spinal cord (Chen et al., 2001) and for the formation of the whisker-related principal sensory nucleus lemniscal pathway (Ding et al., 2003). Both the structural and neurochemical abnormalities occurring at the DRG and spinal cord dorsal horn of *Drg11*-null adult mice and the behavior of these mice in several nociceptive tests pointed to a role for DRG11 in the development of the nociceptive system, namely the establishment of the first link of the ascending nociceptive pathway (Chen et al., 2001). Several aspects remained, however, to be elucidated. Among these stand the clarification of whether DRG11 is also involved in the development of non-nociceptive somatosensory systems and the characterization of the neuronal structures and developmental mechanisms under the control of DRG11.

This study was designed to address the putative function of DRG11 in the development of primary afferent neurons. By looking at markers of large, small peptidergic and small non-peptidergic DRG neurons from the time when sensory neurons are differentiating until adulthood, we conclude that large DRG neurons are not affected by the deletion of the *Drg11* gene, while both peptidergic and non-peptidergic small DRG neurons innervating various peripheral tissues are partially affected. We also show that DRG11 is not required for the specification and survival of primary afferent neurons until birth, neither for the normal innervation of the periphery at this time point. Our observation of decreased numbers of small DRG neurons at P7 together with the marked increase in apoptosis, as compared to wild-type animals, strongly suggests that Drg11 is required for their survival shortly after birth.

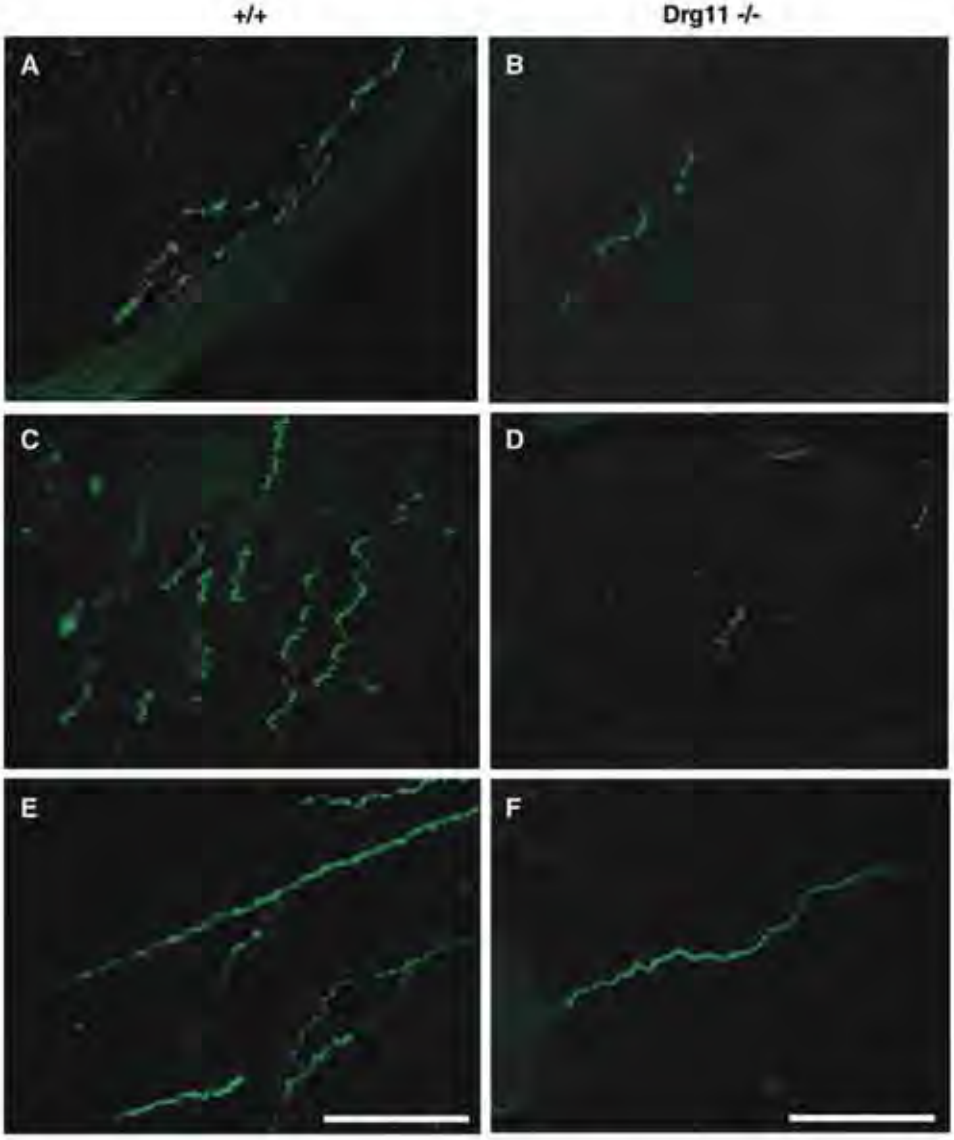

Fig. 5. Cutaneous, visceral and joint CGRP innervation in adult wild-type and *Drg11*$^{-/-}$ mice. Confocal images of CGRP innervation of the epidermis of lumbar skin (A, B), the muscular layer of the urinary bladder (C, D) and the sinovial sheet of the knee joint (E, F). (A, C and E) wild-type mice; (B, D and F) *Drg11*$^{-/-}$ mice. A decrease in staining is apparent in *Drg11*$^{-/-}$ mice as compared to wild-type mice. Scale bars: 100 μm.

Extent of compromise of different sensory neurons in Drg11$^{-/-}$ *mice*

The present data clearly demonstrate that DRG11 is specifically involved in the normal development of an extensive population of small-diameter primary afferent neurons, which are known to convey nociceptive and innocuous thermal input from the periphery. Previous behavior studies have shown that adult *Drg11*$^{-/-}$ mice respond with particularly high thresholds in thermal and mechanical nociceptive tests and present highly reduced nocieceptive behavior in the formalin test (Chen et al., 2001). Although these findings suggested that nociception was markedly disrupted in the absence of DRG11, we now verify that a significant and similar amount of small peptidergic and non-peptidergic primary afferents is still present and innervation is only partially disturbed in all kinds of peripheral tissues. The possibility that the preserved fibers convey mainly thermal innocuous input should be considered. Although data on the response capacity of *Drg11*$^{-/-}$ mice to this kind of stimuli are missing, the size-frequency distribution of DRG neurons in *Drg11*$^{-/-}$ mice (present results) argues against this possibility. Innocuous thermal primary afferent neurons belong mostly in the C unmyelinated group (for review, see Willis and Coggeshall, 1991), and C unmyelinated primary afferents (which, according to Harper and Lawson (1985) have cell bodies measuring between 250 and 500 μm^2) appear to be more affected by *Drg11* deletion than Aδ primary afferents (500 to 750 μm^2; Harper and Lawson, 1985). In *Drg11*$^{-/-}$ mice, IB4 neurons, which comprise mostly small C primary afferents (up to 500 μm^2) (Zwick et al., 2002, present data), were affected along all size range, while CGRP neurons measuring more than 500 μm^2 (A_δ primary afferents) were apparently not affected. Since C fibers make up the bulk of visceral sensory innervation, such a more serious disruption of C primary afferent neurons agrees with the more marked decrease in the amount of fibers stained in the urinary

bladder as compared to the skin or the sinovial sheet. Taken together, our data suggest that DRG11 is required for the normal development of small peptidergic and non-peptidergic primary afferent neurons mainly belonging in the C unmyelinated class.

Large primary afferent neurons were here shown to be maintained in normal amounts, as previously suggested by the normal behavior of *Drg11*$^{-/-}$ mice in the rotarod test. Proprioception and mechanical innocuous processing are therefore likely to be not influenced by DRG11. As to this particular point, *Drg11*$^{-/-}$ mice may constitute a very useful model to study in which extent tactile processing is impaired due to the mis-functioning of the nociceptive system. It should be kept in mind, however, that, according to the present data, these mice are not nociception-null animals. Not only they might be exposed to normal nociceptive input for a short period of their perinatal life, but also nociceptive input conveyed by a few, mainly Aδ fibers may still arrive at the spinal dorsal horn.

The role of DRG11 in the development of the nociceptive system

Although the present data clearly point to a correlation between the nociceptive defects observed in *Drg11*$^{-/-}$ adult mice (Chen et al., 2001) and the disruption of peripheral innervation, the mechanisms underlying the effects of DRG11 in the peripheral sensory system remain to be clarified. Whatever these mechanisms are, it is now clear that DRG11 does not seem to affect the initial generation of the primary nociceptive neurons. Instead, primary afferent neurons appear to fail to survive shortly after birth. This may be due to either a direct effect of DRG11 in the maintenance of a particular primary afferent neuronal population or a failure of these neurons to find their targets in either the spinal dorsal horn or the periphery.

With respect to phenotypic differentiation, it would be very useful to know the molecular basis of the shift of some trkA expressing primary afferent neurons into IB4 expressing neurons at postnatal life. A putative role of DRG11 in this process may partially explain the underrepresentation of non-peptidergic primary afferent neurons in *Drg11*-null mice. However, the decreased number of DRG neurons at P7 together with the observed increase in apoptosis indicates that neuronal death also plays a role in small primary afferent disruption. Curiously, an increase in the numbers of both small and large primary afferent neurons between P0 and P14 was detected not only in wild-type but also, although not so markedly, in knockout mice. Since postnatal neurogenesis has not been reported in the spinal cord (Altman and Bayer, 1984; Horner et al., 2000), it is possible that this increase reflects a higher rate of immunodetection due to augmented expression of neuronal molecules during expansion of peripheral innervation (see Results section). Although disruption of peripheral connectivity cannot be excluded, the possibility that DRG neuronal loss rather results from a failure in establishing synaptic connections with their target neurons in the spinal cord is strongly supported by our findings concerning spinal dorsal horn structural abnormalities. *Drg11*$^{-/-}$ mice present a distorted dorsal horn which, in Nissl staining, appears to lack the small size neuronal population of the substantia gelatinosa (Chen et al., 2001). In accordance, PKCγ staining is completely absent in *Drg11*$^{-/-}$ mice, while CGRP and trkA immunostaining is markedly reduced and shifted medially as compared to wild-type mice (Chen et al., 2001). Golgi impregnation of adult *Drg11*$^{-/-}$ mice spinal cord revealed a massive disappearance of lamina II neurons and a very marked reduction in numbers of lamina I neurons (unpublished data). It is therefore likely that DRG11 deficiency-dependent reduction of peptidergic and non-peptidergic primary afferent innervation is secondary to a primary effect upon differentiation of nociceptive spinal neurons located in laminae I and II.

It should be taken in mind that nociceptive thin myelinated fibers (Aδ) also innervate lamina V, in the deep dorsal horn (Light and Perl, 1979a,b; Cervero and Connell, 1984). Although a comprehensive evaluation of the extent of compromise of spinal neuronal differentiation due to the lack of DRG11 is missing, neither studies based on Nissl (Chen et al., 2001) or Golgi (unpublished data) staining of the *Drg11*$^{-/-}$ spinal dorsal horn nor labeling for various spinal cord markers (Chen et al., 2001) point to a defect in the deep dorsal horn. It is therefore possible that the population of spared, mainly Aδ small size primary afferent neurons is the one that projects to the deep dorsal horn. This being the case, *Drg11*$^{-/-}$ mice are still able to undergo nociceptive processing through the deep dorsal horn, although in the absence of the modulatory effects of substantia gelatinosa (lamina II). Furthermore, the spinothelencephalic circuit which originated in lamina V, which was recently shown to receive input from lamina II (Braz et al., 2005), would be affected. In this light and taking into account the putative role of the deep dorsal horn in processing sustained pain (Abbadie and Besson, 1992), it would be interesting to analyze how *Drg11* knockout mice behave in chronic pain conditions.

Experimental methods

Genotyping and maintenance of animals

Wild-type mice and mice in which the gene *Drg11* was deleted following homologous recombination were used. The ethical guidelines for investigation of experimental pain in animals (Zimmermann, 1983) and the European Community Council Directive of 24 November 1986 (86/609/EEC) were followed.

The design of the targeting construct deleted exons 3 and 4, which encode most of the paired-like homeodomain, replacing the putative DNA binding region by IRES-Tau-lacZ-neo cassette.

Drg11$^{-/-}$ mice and their wild-type littermates were genotyped as previously described (Chen et al., 2001). In brief, a tail sample was taken from each mouse and lysed in buffer (100 mM Tris–HCl, pH 8.8, 0.2% SDS, 200 mM NaCl, 1 mM $CaCl_2$ and 100 μg/ml proteinase K) overnight. The extracted DNA was precipitated with isopropanol and resuspended in 50 μl of water for at least 2 h at 65 °C. PCR analysis was performed using a set of neo primers and a set of primers targeting the deleted region of the mutant allele (5′ TGC,AAA,GCA,AAT,CTG,ACC,GCT,CTG 3′ and 5′ GAA,CAG,AAA,CAG,CAT,GGA,GGA,AAC 3′). The *Drg11*$^{-/-}$ mice were bred and maintained at the IBMC animal facility. The day when the vaginal plug was formed was considered the embryonic day 0.5 (E0.5).

Tissue preparation

Four wild-type and four knockout animals per embryonic and postnatal age were analyzed at a total of 48 animals.

Embryos (E14.5 and E18.5) were removed by caesarian surgery of pregnant females under sodium pentobarbital anesthesia (50 mg/kg i.p.) and immediately fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. After 6–8 h in the fixative, embryos were immersed in a 30% sucrose solution overnight for cryoprotection and then cut on a cryostat at 12 μm.

Mice at postnatal ages were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The urinary bladder was dissected and animals were then perfused through the ascending aorta with 5 ml of phosphate-buffered

saline 0.1 M (PBS) followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The skin of the lumbar region and plantar hind limbs, the knee joint sinovial sheet and the L3 and L4 DRGs were removed. All tissue samples, except the knee joint sinovial sheet, were immersed immediately after dissection in the fixative for 2 h. The sinovial sheets were washed in PBS for 1 h, post-fixed in the same fixative for another hour and then treated with ethanol at 10%, 25%, 40%, 25%, 10%, 5 min each, to facilitate antibody penetration (Just et al., 2001). All tissue samples, except the sinovial sheets, were immersed in 30% sucrose in phosphate buffer overnight for cryoprotection. Cryostat sections were obtained from the skin and the DRGs at 10 μm and from the bladder trigone at 20 μm. The sinovial sheets were mounted *in toto* in PBS/glycerol (1:3).

Nissl staining

All sections of one L3 and one L4 DRGs taken from one animal per age E14.5, E18.5, P0, P7, P14 and P21–P30 (adulthood) were used for Nissl staining. Briefly, sections were stained with 0.5% cresyl violet for 15 min, rinsed in tap water, dehydrated and mounted in Eukitt®.

Immunohistochemical staining

One in every five sections from the remaining L3 and L4 DRGs, the lumbar and plantar skin and the bladder trigone were incubated for 1 h in 10% normal goat serum (NGS) in PBS followed by immersion for 24–36 h in either a monoclonal antibody against NF200 (E14.5, E18.5, P0, P7, P14 and adulthood) raised in mouse (Sigma, 1:500), an antiserum against trkA (Chemicon, 1:3000) (E14.5 and E18.5) raised in rabbit, an antiserum against CGRP (P0, P7, P14 and adulthood) raised in rabbit (Peninsula Laboratories Europe, UK, 1:8000) or IB4 (P0, P7, P14 and adulthood) (Sigma, UK; biotinylated at a dilution of 10 μg/ml). The sinovial sheets stayed for 2 h in a blocking solution containing bovine serum albumin (BSA) and PBST before incubation in the primary antibodies described above. Sections were then washed in PBST and incubated for 1 h, at room temperature, in the appropriate secondary antibody, namely: MOM kit for detecting NF200 (Vector Laboratories, PK-2200); biotinylated swine anti-rabbit antiserum for detecting trkA and CGRP in the DRGs (Dakopatts, Dako A/5, Copenhagen, Denmark, 1:200); goat anti-rabbit Alexa Fluor 488 for detecting CGRP in peripheral tissues (Molecular Probes Europe, Netherlands, 1:1000); streptavidin Alexa 488 for detecting IB4 (Molecular Probes Europe, Netherlands, 1:1000). Sections were rinsed in PBS, mounted with PBS/glycerol and viewed under BioRad confocal microscope.

Omitting the primary antibodies in the reaction resulted in light background staining but no staining of neuronal profiles.

For apoptosis detection, immunohistochemistry was performed on L3 and L4 DRG sections from P0 and P7 animals with a rabbit antibody against anti-cleaved caspase-3 (Cell Signaling Technologies, 1:200) using the avidin–biotin–immunoperoxidase technique (Vectastain Elite avidin–biotin–immunoperoxidase kit reagents from Vector Labs, Burlingame, CA). Before incubation with the primary antibody, sections were subjected to heat-induced antigen retrieval by incubation in a Citrate buffer, 2 g/l at pH 6.0 for 15 min. Double-labeling immunofluorescence was performed on L3 and L4 DRG sections from P7 animals using the same caspase antibody (1:400) and an antibody against peripherin (Santa Cruz, Biotechnology Inc., 1:50) and Alexa fluorochrome secondary antibodies.

Stereological counting of DRG neurons

The number of neurons stained by Nissl, immunoreactive for NF200 or CGRP or stained for IB4 at L3 and L4 DRGs was calculated by stereological methods using a systematic random sampling procedure (Gundersen and Jensen, 1987). The number of neurons was calculated by multiplying the ganglion volume by cell density following the formula of De Hoff and Rhines (Weibel, 1979). The volume of each ganglion was estimated following the principle of Cavalieri (Cavalieri, 1966) for a section thickness of 12 μm, as determined by the cryostat. The area occupied by the ganglion in each section was estimated by counting all points of a two-dimension frame superimposed to the computer screen, at a total magnification of 400× (Krekule and Gundersen, 1989). All sections of each ganglion, at an average of 12 sections per ganglion, were studied. Neuronal density was calculated from the number of neurons that fell within a predefined area of a two-dimension rectangular counting frame (Gundersen and Jensen, 1987).

Cell area measurements and frequency distribution analysis

Using the same predefined rectangular frame mentioned above, the cross-section area of one hundred cells taken from random sections of L3 and L4 adult ganglia and showing clear nuclear profiles was measured. The cells' outline was drawn and the software ImageJ, version 1.33 used to automatically calculate the cell area. The data were represented as cell area frequency histograms.

Quantification of apoptotic cells

The number of caspase-3-immunoreactive DRG cells, expressed as mean ± SEM, was determined by counting the immunoreactive neuronal profiles in 10 sections randomly taken from L3 to L4 DRGs from three wild-type and three Drg11$^{-/-}$ mice at the age P7.

Quantification of peripheral tissue innervation

Images of CGRP immunoreactivity or IB4 binding in the lumbar and plantar skin, the bladder trigone and the sinovial sheet were digitalized from three randomly taken, non-contiguous sections per animal and converted in black and white images with Adobe PhotoShop 5.5 (Buwalda et al., 1997). The immunoreactive area was evaluated by the number of pixels generated by immunoreactive fibers in each image. Pixels were counted using Visual Basic custom-made software running on a PC (Avelino et al., 2002).

Data analysis

Statistical analysis was performed using the Student's *t* test corrected for multiple comparisons. The number of DRG cells stained for the various markers or the number of pixels generated by fibers immunoreactive for CGRP or IB4 was compared between wild-type and *Drg11*$^{-/-}$ mice. The number of stained DRG cells in either wild-type or knockout mice was also compared between adjacent time points. The accepted level of significance in all tests was $p \leq 0.05$. Data are indicated as mean ± SEM.

Acknowledgments

The authors would like to gratefully acknowledge Profs. Dulce Madeira and Manuel Paula-Barbosa for their assistance in the use of stereological methods, Stephan Just for technical advice regarding dissection of the knee joint capsule and Luis Mendonça for Visual Basic programming. This work was supported by FCT grants no. PRAXIS XXI/BD/11519/97 and no. POCTI/1999/NSE/32357.

References

Abbadie, C., Besson, J.-M., 1992. C-*fos* expression in the rat lumbar spinal cord during the development of adjuvant-induced arthritis. Neuroscience 48, 985–993.

Altman, J., Bayer, S.A., 1984. The development of rat spinal cord. Adv. Anat., Embryol. Cell Biol. 85, 1–168.

Alvarez, F.J., Morris, H.R., Priestley, J.V., 1991. Sub-populations of smaller diameter trigeminal primary afferent neurons defined by expression of calcitonin gene-related peptide and the cell surface oligosaccharide recognized by monoclonal antibody LA4. J. Neurocytol. 20, 716–731.

Ambalavanar, R., Moritani, M., Haines, A., Hilton, T., Dessem, D., 2003.

Chemical phenotypes of muscle and cutaneous afferent neurons in the rat trigeminal ganglion. J. Comp. Neurol. 460, 167–179.

Avelino, A., Cruz, C., Nagy, I., Cruz, F., 2002. Vanilloid receptor 1 expression in the rat urinary tract. Neuroscience 109, 787–798.

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F., Priestley, J.V., 1995. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur. J. Neurosci. 7, 1484–1494.

Bennett, D.L.H., Averill, S., Clary, D.O., Priestley, J.V., McMahon, S.B., 1996a. Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. Eur. J. Neurosci. 8, 2204–2208.

Bennett, D.L.H., Dmietrieva, N., Priestley, J.V., Clary, D.O., McMahon, S.B., 1996b. trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. Neurosci. Lett. 206, 33–36.

Bennett, D.L.H., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B., Priestley, J.V., 1998. A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J. Neurosci. 18, 3059–3072.

Bennett, D.L.H., Boucher, T.J., Armanini, M.P., Poulsen, K.T., Michael, G.J., Priestley, J.V., Philips, H.S., McMahon, S.B., Shelton, D.L., 2000. The glial cell line-derived neurotrophic factor family receptor components are differentially regulated within sensory neurons after nerve injury. J. Neurosci. 20, 427–437.

Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005. Parallel "Pain" pathways arise from subpopulations of primary afferent nociceptor. Neuron 47, 787–793.

Buwalda, J., Colnot, D.R., Bleys, R.L.A.W., Groen, G.J., Thrasivoulou, C., Cowen, T., 1997. Imaging and analysis of perivascular nerves in human mesenteric and coronary arteries: a comparison between epi-fluorescence and confocal microscopy. J. Neurosci. Methods 73, 129–134.

Cavalieri, B., 1966. Geometria degli Indivisibili. Unione Tipografico Editrice, Torrino.

Cervero, F., Connell, L.A., 1984. Fine afferent fibers from viscera do not terminate in the substantia gelatinosa of the thoracic spinal cord. Brain Res. 294, 370–374.

Chen, Z.-F., Rebelo, S., White, F., Malmberg, A.B., Baba, H., Lima, D., Woolf, C.J., Basbaum, A.I., Anderson, D.J., 2001. The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. Neuron 31, 59–73.

Coggeshall, R.E., Pover, C.M., Fitzgerald, M., 1994. Dorsal root ganglion cell death and surviving cell numbers in relation to the development of sensory innervation in the rat hindlimb. Dev. Brain Res. 82, 193–212.

Coimbra, A., Sodré-Borges, B.P., Magalhães, M.M., 1974. The substantial gelatinosa of Rolandi of the rat. Fine structure, cytochemistry (acid phosphatase) and changes after dorsal root section. J. Neurocytol. 3, 199–217.

Ding, Y.-Q., Yin, J., Xu, H.-M., Jacquin, M.F., Chen, Z.-F., 2003. Formation of whisker-related principal sensory nucleus-based lemniscal pathway requires a paired homeodomain transcription factor, *Drg11*. J. Neurosci. 23, 7246–7254.

Ding, Y.-Q., Yin, J., Kania, A., Zhao, Z.-Q., Johnson, R.L., Chen, Z.-F., 2004. Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. Development 131, 3693–3703.

Ebinger, M., Schmidt, R.F., Heppelmann, B., 2001. Composition of the medial and posterior articular nerves of the mouse knee joint. Somatosens. Motor Res. 18, 62–65.

Fariñas, I., Yoshida, C.K., Backus, C., Reichardt, L.F., 1996. Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron 17, 1065–1978.

Fariñas, I., Cano-Jaimez, M., Bellmunt, E., Soriano, M., 2002. Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res. Bull. 57, 809–816.

Fitzgerald, M., 2005. The development of nociceptive circuits. Nat. Rev., Neurosci. 6, 507–520.

Fitzgerald, M., Fulton, B.P., 1992. The physiological properties of developing sensory neurons. In: Scott, S.A. (Ed.), Sensory Neurons: Diversity, Development and Plasticity. Oxford Univ. Press, NY, pp. 287–306.

Fundin, B.T., Arvidsson, J., Aldskogius, H., Johansson, O., Rice, S.N., Rice, F.L., 1997. Comprehensive immunofluorescence and lectin binding analysis of intervibrissal fur innervation in the mystacial pad of the rat. J. Comp. Neurol. 385, 185–206.

Gundersen, H.J.G., Jensen, E.B., 1987. The efficiency of systematic sampling in stereology and its prediction. J. Microsc. 147, 229–263.

Hall, A.K., Ai, X., Hickman, G.E., MacPhedran, S.E., Nduaguba, C.O., Robertson, C.P., 1997. The generation of neuronal heterogeneity in a rat sensory ganglia. J. Neurosci. 17, 2775–2784.

Hanesch, U., Heppelmann, B., Schmidt, R.F., 1991. Substance P- and calcitonin gene-related peptide immunoreactivity in primary afferent neurons of the cat's knee joint. Neuroscience 45, 185–193.

Harper, A.A., Lawson, S.N., 1985. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. J. Physiol. 359, 31–46.

Heppelmann, B., Shahbazian, Z., Hanesch, U., 1997. Quantitative examination of calcitonin gene-related peptide immunoreactive nerve fibres in the cat knee joint capsule. Anat. Embryol. 195, 525–530.

Horner, P.J., Power, A.E., Kempermann, G., Kuhn, H.G., Palmer, T.D., Winkler, J., Thal, L.J., Gage, F.H., 2000. Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. J. Neurosci. 20, 2218–2228.

Hunt, S.P., Mantyh, P.W., 2001. The molecular dynamics of pain control. Nat. Rev., Neurosci. 2, 83–91.

Ivanavicius, S.P., Blake, D.R., Chessell, I.P., Mapp, P.I., 2004. Isolectin B4 binding neurons are not present in the rat knee joint. Neuroscience 128, 555–560.

Jackman, A., Fitzgerald, M., 2000. Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. J. Comp. Neurol. 418, 281–298.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev., Genet. 1, 20–29.

Just, S., Leipold-Büttner, C., Heppelmann, B., 2001. Histological demonstration of voltage dependent calcium channels on calcitonin gene-related peptide-immunoreactive nerve fibres in the mouse knee joint. Neurosci. Lett. 312, 133–136.

Kirstein, M., Fariñas, I., 2002. Sensing life: regulation of sensory neuron survival by neurotrophins. Cell. Mol. Life Sci. 59, 1787–1802.

Kitao, Y., Robertson, B., Kudo, M., Grant, G., 1996. Neurogenesis of subpopulations of rat lumbar dorsal root ganglion neurons including neurons projecting to the dorsal column nuclei. J. Comp. Neurol. 371, 249–257.

Krekule, I., Gundersen, H.J.G., 1989. Computer graphics enhancement of measurements in stereology. Acta Stereol. 8/2, 533–535.

Lawson, S.N., 1992. Morphological and biochemical cell types of sensory neurons. In: Scott, S.A. (Ed.), Sensory Neurons: Diversity, Development and Plasticity. Oxford Univ. Press, NY, pp. 27–59.

Lawson, S.N., Biscoe, T.J., 1979. Development of mouse dorsal root ganglia: an autoradiographic and quantitative study. J. Neurocytol. 8, 265–274.

Lawson, S.N., Waddell, P.J., 1991. Soma neurofilament immunoreactivity is related to cell size and fiber conduction velocity in rat primary sensory neurons. J. Physiol. 435, 41–63.

Light, A.R., Perl, E.R., 1979a. Reexamination of the dorsal root projection to the spinal dorsal horn including observation on the differential termination of coarse and fine fibers. J. Comp. Neurol. 186, 117–132.

Light, A.R., Perl, E.R., 1979b. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J. Comp. Neurol. 186, 133–150.

Markus, A., Patel, T.D., Snider, W.D., 2002. Neurotrophic factors and axonal growth. Curr. Opin. Neurobiol. 12, 523–531.

Maro, G.S., Vermeren, M., Voiculescu, O., Melton, L., Cohen, J., Charnay, P., Topilko, P., 2004. Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat. Neurosci. 7, 930–938.

McCarthy, P.W., Lawson, S.N., 1990. Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. Neuroscience 34, 623–632.

Michael, G.J., Averill, S., Nitkunan, A., Rattray, M., Bennett, D.L.H., Yan, Q., Priestley, J.V., 1997. Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in trkA-expressing dorsal root ganglion cells and their central terminations within the spinal cord. J. Neurosci. 17, 8476–8490.

Molliver, D.C., Snider, W.D., 1997. Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. J. Comp. Neurol. 381, 428–438.

Molliver, D.C., Radeke, M.J., Feinstein, S.C., Snider, W.D., 1995. Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J. Comp. Neurol. 361, 404–416.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., Snider, W.D., 1997. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19, 849–861.

Nagy, J.I., Hunt, S.P., 1982. Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience 7, 89–97.

Orozco, O.E., Walus, L., Sah, D.W.Y., Pepinsky, R.B., Sanicola, M., 2001. GFRalpha3 is expressed predominantly in nociceptive sensory neurons. Eur. J. Neurosci. 13, 2177–2182.

Rebelo, S., Chen, Z.-F., Anderson, D.J., Lima, D., 2001. The role of DRG11 in sensory enervation in Drg11 knockout mice. Program No. 618.7 2001 Abstract Viewer/Itinerary planner. Society for Neuroscience, San Diego. Online.

Reynolds, M.L., Fitzgerald, M., Benowitz, L.I., 1991. GAP-43 expression in developing cutaneous and muscle nerves in the rat hindlimb. Neuroscience 41, 201–211.

Saito, T., Greenwood, A., Sun, Q., Anderson, D.J., 1995. Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol. Cell. Neurosci. 6, 280–292.

Silos-Santiago, I., Molliver, D.C., Ozaki, S., Smeyne, R.J., Fagan, A.M., Barbacid, M., Snider, W.D., 1995. Non-TrkA-expressing small DRG neurons are lost in *TrkA* deficient mice. J. Neurosci. 15, 5929–5942.

Silvermann, J.D., Kruger, L., 1990. Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. J. Neurocytol. 19, 789–801.

Snider, W.D., McMahon, S.B., 1998. Tackling pain at the source: new ideas about nociceptors. Neuron 20, 629–632.

Snider, W.D., Silos-Santiago, I., 1996. Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. Philos. Trans. R. Soc. London Ser. B 351, 395–403.

Streit, W.J., Schulte, B.A., Balentine, J.D., Spicer, S.S., 1986. Evidence for glycoconjugate in nociceptive primary sensory neurons and its origin from the Golgi complex. Brain Res. 377, 1–17.

Wang, H., Rivero-Melián, C., Robertson, B., Grant, G., 1994. Transganglionic transport and binding of the isolectin B4 from *Griffonia simplicifolia* I in rat primary sensory neurons. Neuroscience 62, 539–551.

Weibel, E.R., 1979. Stereological Methods, vol. 1, Practical Methods for Biological Morphometry. Academic Press, London.

White, F.A., Silos-Santiago, I., Molliver, D.C., Nishimura, M., Phillips, H., Barbacid, M., Snider, W.D., 1996. Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. J. Neurosci. 16, 4662–4672.

Willis, W.D., Coggeshall, R.E., 1991. Sensory Mechanisms of the Spinal Cord. Plenum Press, NY.

Yuan, J., Yankner, B.A., 2000. Apoptosis in the nervous system. Nature 407, 802–809.

Zhuang, B., Sockanathan, S., 2006. Dorsal–ventral patterning: a view from the top. Curr. Opin. Neurobiol. 16, 20–24.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.

Zwick, M., Davis, B.M., Woodbury, J., Burkett, J.N., Koerber, H.R., Simpson, J.F., Albers, K.M., 2002. Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J. Neurosci. 22, 4057–4065.

Zylka, M.J., Rice, F.L., Anderson, D.J., 2005. Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45, 17–25.

DEVELOPMENTAL DYNAMICS 239:1684–1694, 2010

RESEARCH ARTICLE

Prrxl1 is Required for the Generation of a Subset of Nociceptive Glutamatergic Superficial Spinal Dorsal Horn Neurons

Sandra Rebelo, Carlos Reguenga, Claúdia Lopes, and Deolinda Lima*

Perception of noxious events relies on activation of complex central neuronal circuits. The spinal cord dorsal horn plays a pivotal role in the process relaying to the brain various types of somatosensory input. These functions are accomplished by distinct sensory neurons specifically organized in different laminae. They differentiate during development in a spatial-temporal order due to the expression of combinatorial sets of homeodomain transcription factors. Here we demonstrate that the differential expression of the homeodomain transcription factors Prrxl1 (DRG11), Tlx3, and Lmx1b defines various subpopulations of spinal cord dorsal horn glutamatergic early born and late born neurons. Accordingly, in the superficial dorsal horn of *Prrxl1*$^{-/-}$ mice, the number of glutamatergic neurons is reduced by 70%, while the number of Golgi-impregnated and noxious-induced Fos immunoreactive neurons is reduced by 85%. These results suggest a crucial role for Prrxl1 in the generation of various subpopulations of nociceptive glutamatergic superficial dorsal horn neurons. *Developmental Dynamics 239:1684–1694, 2010.* © 2010 Wiley-Liss, Inc.

Key words: Prrxl1; Drg11; spinal cord; pain; *c-fos*; differentiation; embryonic development; nociception; glutamatergic neurons; substantia gelatinosa

Accepted 25 March 2010

INTRODUCTION

Perception of sensory information by the brain requires highly ordered synaptic connectivity between peripheral sensory neurons and their targets in the central nervous system. Somatosensory information is processed in the spinal cord dorsal horn, which is responsible for modulating, integrating, and relaying to higher brain centers various types of sensory input using a complex circuit that is established during embryonic development (for review, see Gillespie and Walker, 2001; Julius and Basbaum, 2001). The molecular events that govern the generation of specific types of dorsal horn sensory neurons along the neural tube began to be unmasked only recently. Sensory neurons are born and start to migrate towards their final location along the dorso-ventral axis in a highly spatial and temporal order (Goulding et al., 2002; Caspary and Anderson, 2003; Helms and Johnson, 2003) due to the expression of multiple patterning genes that encode a set of homeodomain transcription factors in a combinatorial manner (Lee and Jessell, 1999; Jessell, 2000; Goulding et al., 2002; Müller et al., 2002; Helms et al., 2005). These genes are important to instruct neurons to fulfill their differentiation program (Chen et al., 2001; Müller et al., 2002; Qian et al., 2002, Zhou and Anderson, 2002; Cheng et al., 2004; Ding et al., 2004).

In the mouse spinal cord, dorsal horn neurons arise from progenitors in the ventricular zone in two neurogenic waves between embryonic days 10 and 14 (E10–14). The first wave takes place between E10.5 and E11.5 and generates six subpopulations (dI1–6) of early-born neurons that will populate the deep dorsal horn. The second wave (E12–14.5) originates two late-born neuronal populations (dILA and dILB), which migrate dorsally and form the superficial dorsal horn (Gross et al., 2002; Müller et al., 2002). The early-born neurons can be

Additional Supporting Information may be found in the online version of this article.

Laboratório de Biologia Celular e Molecular da Faculdade de Medicina da Universidade do Porto and Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Portugal

Grant sponsor: FCT; Grant number: POCI/SAU-NEU/59093/2004.

***Correspondence to: Deolinda Lima, Laboratório de Biologia Celular e Molecular, Faculdade de Medicina da Universidade do Porto, Alameda Hernani Monteiro, 4200-319 Porto, Portugal. E-mail: limad@med.up.pt**

DOI 10.1002/dvdy.22305
Published online 23 April 2010 in Wiley InterScience (www.interscience.wiley.com).

subdivided into two major classes: Class A (dI1–3) neurons are born in the dorsal alar plate, depend on roof plate signals, and are Lbx1-independent (Liem et al., 1997; Lee et al., 1998, 2000; Wine-Lee et al., 2004); Class B neurons (dI4–6) arise from the ventral alar plate, are independent of roof plate signals, and are Lbx1-dependent (Pierani et al., 2001; Gross et al., 2002; Matise, 2002; Müller et al., 2002; Cheng et al., 2004). dI3 and dI5 neurons express the glutamatergic fate determinant Tlx3 (Cheng et al., 2004). dI1–3 neurons are thought to be involved in proprioceptive processing (Bermingham et al., 2001; Gowan et al., 2001). As to late-born neurons, dILA neurons express Pax2, Lhx1/5, and Ptf1a, and dILB neurons are Tlx1/3-positive and Lmx1b-positive (Gross et al., 2002; Müller et al., 2002; Cheng et al., 2005). Glutamatergic neurons differentiate from the dILB population, while dILA neurons follow the GABAergic fate (Gross et al., 2002; Müller et al., 2002; Cheng et al., 2005; Glasgow et al., 2005).

Prrxl1 (also known as Drg11) is a paired-like homeodomain protein that is expressed both in early-born (dI3 and dI5) and glutamatergic late-born (dILB) neurons (Rebelo et al., 2007). Mice with deletions in *Prrxl1* gene present defects in the spinal cord superficial dorsal horn similar to those observed in *Tlx3/1* or *Lmx1b* knockout mice. This is suggestive that the three transcription factors take part in the genetic cascade involved in building up the superficial spinal cord neuronal circuitry (Chen et al., 2001; Gross et al., 2002; Müller et al., 2002; Qian et al., 2002). The specific role of these genes remains, however, to be characterized. In *Prrxl1* knockout mice (*Prrxl1*$^{-/-}$), abnormalities in DRG spinal projections, superficial dorsal horn structure and neurochemistry, and nociceptive responses have been identified (Chen et al., 2001). Recent data point to a role for *Prrxl1* in early postnatal survival of normally differentiated small-size primary afferent neurons innervating various kinds of peripheral tissues, which would explain the nociceptive deficits observed in *Prrxl1*-null mutant mice (Rebelo et al., 2006).

This study investigates the role of Prrxl1 in the specification of nociceptor targets in the spinal superficial dorsal horn. Taking advantage of multiple-labeling combining immunohistochemical detection of Prrxl1 and the two functionally related transcription factors, Tlx3 and Lmx1b, which are likely to be upstream to Prrxl1 (Ding et al., 2004), we were able to define various subsets of early- and late-born neuronal populations. Moreover, in perinatal *Prrxl1*$^{-/-}$ mice, expression of Tlx3 and Lmx1b in the spinal cord superficial dorsal horn was deeply reduced, the preserved neurons equaling in number those that did not express Prrxl1 in the wild-type mice. Golgi studies confirmed the absence of a large amount of neurons in laminae I–III of *Prrxl1*$^{-/-}$ mice but not in the deep dorsal horn. Accordingly, the number of neurons *c-fos* activated after cutaneous and visceral noxious stimulation was reduced but only in the superficial dorsal horn, suggesting that the remaining neurons are not functionally affected by the loss of Prrxl1-dependent neurons.

RESULTS

Migration Pattern of Glutamatergic Neurons in the Developing Spinal Cord

Previous immunohistochemical studies showed that Prrxl1 is detected in mice from the time of neural tube closure (E10.5) up to P21, becoming restricted to the spinal cord superficial dorsal horn (Rebelo et al., 2007). Two functionally related transcription factors, Tlx3 and Lmx1b, have expression patterns similar to Prrxl1 and co-localize with VGLUT2, which was taken as indicative of their involvement in glutamatergic specification (Cheng et al., 2004). To better define the role of Prrxl1 in the differentiation of the glutamatergic neuronal population, a time course analysis of the expression of Prrxl1 and either Tlx3 or Lmx1b was performed using double-labeling immunohistochemistry (Fig. 1). At E10.5, Prrxl1 was exclusively present in the dI3 and dI5 early-born neuronal subpopulations, as confirmed by the absence of co-localization with Lim1/2, a marker of dI2, dI4, and dI6 interneurons (Fig. 1A, H). Prrxl1-positive neurons lied adjacent to but outside the ventricular zone, indicating that they are postmitotic neurons. Tlx3 marked dI3 and dI5 neurons (Fig. 1A), while Lmx1b marked dI5 neurons exclusively (Fig. 1H). Prrxl1 co-localized extensively both with Tlx3 (Fig. 1A) and Lmx1b (Fig. 1H). At E11.0, both dI3 and dI5 Prrxl1-expressing neurons started the migration towards the neural tube (Rebelo et al., 2007), but a few dI5 neurons appeared to form streams that extended to the ventral neural tube (Fig. 1B–F, I–M; small arrows). At E12.0 and E12.5, numerous newly formed (late-born) neurons were concentrated adjacent to the ventricular zone and along a stream in lateral migration (Fig. 1C,D,J,K; large arrows). Between E14.5 and E16.5, neurons assumed their final position in the superficial dorsal horn (Fig. 1E,F,L,M). At these ages, as well as at P7 (Fig. 1G,N), Prrxl1-positive neurons were mainly located in lamina II (Tables 1–3; see also Supp. Fig. S1, which is available online) and co-expressed extensively either Tlx3 (Fig. 1E–G; Table 1) or Lmx1b (Fig. 1L–N; Table 3).

Altogether, these data unveil a highly dynamic synchrony in the migration of neurons expressing Prrxl1, Tlx3, and Lmx1b, and show a high degree of co-expression between the three transcription factors along development of glutamatergic neurons.

Co-expression of Prrxl1, Tlx3, and Lmx1b Defines Subsets of Glutamatergic Superficial Dorsal Horn Neurons

In order to ascertain whether co-expression of the various transcription factors identifies different categories among dorsal horn glutamatergic neurons, counting of early-born and late-born neurons was performed at E10.5 and P7, respectively. It should be noted that staining intensity varied considerably, suggesting differential levels of expression for each transcription factor. Nevertheless, all labeled neurons were considered for counting irrespective of their staining level.

Early-born neurons.

Prrxl1-positive neurons were present in similar numbers in dI3 and dI5 regions (Fig. 2B,D). As expected from the results obtained by Müller et al.

(2002) and Cheng et al. (2005), the entire dI5 Prrxl1-positive neuronal population co-expressed the dI4–dI6 marker, Lbx1 (Fig. 2A).

All Prrxl1-positive neurons present in both the dI3 and dI5 regions also expressed Tlx3 (Fig. 2B,B′,B″). However, a large subpopulation of Tlx3-positive neurons in both domains did not co-localize with Prrxl1 (Fig. 2B,B′,B″). dI3 glutamatergic neurons fell into two subpopulations: one expressing only Tlx3 (Prrxl1$^-$/Tlx3$^+$, 66%; Fig. 2E) and another expressing both Prrxl1 and Tlx3 (Prrxl1$^+$/Tlx3$^+$, 34%; Fig. 2E).

In the dI5 region, Tlx3-positive neurons also co-expressed Lmx1b extensively (Fig. 2C,C″). Since, in addition, some Prrxl1 neurons did not express Lmx1b (Fig. 2D,D″) and all Lmx1b-positive neurons co-expressed Tlx3 (Fig. 2C,C″), three distinct dI5 neuronal populations were identified: a prevalent one, expressing Tlx3 and Lmx1b (Prrxl1$^-$/Tlx3$^+$/Lmx1b$^+$, 50%), and two other identical in number expressing either Prrxl1 and Tlx3 (Prrxl1$^+$/Tlx3$^+$/Lmx1b$^-$, 23%) or Prrxl1, Tlx3, and Lmx1b (Prrxl1$^+$/Tlx3$^+$/Lmx1b$^+$, 27%) (Fig. 2F).

Late-born neurons.

It is known that by E14.5, neurogenesis in the dorsal spinal cord has already ceased (Nornes and Carry, 1978). From this time point on, dorsal spinal cord neurons mature and start to populate the dorsal horn at their final position in the various laminae that constitute the spinal dorsal horn (Müller et al., 2002). Counting of late-born neurons was performed at P7 because this age has the advantage of presenting the dorsal spinal cord neatly laminated and expressing Prrxl1 at levels high enough to allow its detection (Rebelo et al., 2007).

Prrxl1-positive neurons spanned the entire superficial dorsal horn from laminae I to III (Fig. 3A,B,D; Tables 1 and 3) and co-localized extensively with Lbx1 in lamina III (Fig. 3A), being extremely scattered in the deep dorsal horn (lamina IV–V, 5 neurons on average; 1.8% of all Prrxl1-expressing neurons; Fig. 3A,B,D). They were more abundant in lamina II (Tables 1 and 3; chart in Fig. 3), where they amounted to 65% of all Prrxl1-immunoreactive neurons (Supp. Fig. S1). The distribution per lamina of Tlx3- and Lmx1b-positive neurons followed the same pattern (Tables 1–3; chart in Fig. 3; Supp. Fig. S1).

A large amount of Prrxl1-positive neurons co-expressed Tlx3 (81%, Table 1) or Lmx1b (89%, Table 3) (Fig. 3B, D). Tlx3 neurons (Fig. 3B) and Lmx1b neurons (Fig. 3D) co-expressed Prrxl1 in about 60 to 70% in all superficial dorsal horn laminae (Tables 1 and 3; chart in Fig. 3). Considering that all Tlx3-positive neurons also expressed Lmx1b (Fig. 3C; Table 2; Dai et al., 2008), four distinct neuronal populations could be identified (Fig. 3E). Neurons expressing Prrxl1 contributed altogether to 74% of the entire superficial dorsal horn glutamatergic neuronal population and belonged in three categories (Fig. 3E): neurons expressing Prrxl1, Tlx3, and Lmx1b (Prrxl1$^+$/Tlx3$^+$/Lmx1b$^+$, 58%); neurons expressing both Prrxl1 and Lmx1b (Prrxl1$^+$/Tlx3$^-$/Lmx1b$^+$, 7%); and neurons expressing only Prrxl1 (Prrxl1$^+$/Tlx3$^-$/Lmx1b$^-$, 8%). The remaining 27% neurons were Prrxl1-negative and expressed both Tlx3 and Lmx1b (Prrxl1$^-$/Tlx3$^+$/Lmx1b$^+$) (Fig. 3E). Notably, neurons co-localizing Tlx3 and Lmx1b with or without Prrxl1 amounted to 85% of all superficial dorsal horn glutamatergic neurons (Fig. 3E) and predominated in laminae I–II (Fig. 3C), whereas Lmx1b neurons that did not express Tlx3 were concentrated in lamina III (Fig. 3C). This lamina III population is likely to correspond to the Prrxl1$^+$/Tlx3$^-$/Lmx1b$^+$ neurons since Prrxl1 neurons that did not co-express Tlx3 in lamina III (Fig. 3B) were much more abundant (40 in Table 1) than those that did not co-express Lmx1b (Prrxl1$^+$/Tlx3$^-$/Lmx1b$^-$, 4 in Table 3; Supp. Fig. S2). Moreover, the Prrxl1$^+$/Tlx3$^-$/Lmx1b$^-$ subpopulation prevailed in lamina II since Prrxl1-positive neurons non-expressing either Tlx3 (Table 1) or Lmx1b (Table 3) were similar in number and concentrated in lamina II (84%; Supp. Fig. S2).

Defective Development of Superficial Dorsal Horn Glutamatergic Neurons in Prrxl1 Mutant Mice

In the spinal cord of *Prrxl1*$^{-/-}$ embryos, the differentiation and migration of superficial dorsal horn neurons expressing Tlx3 or Lmx1b occurred normally until E14.5, in accordance with the results of Chen et al. (2001). From E18.5 on, a progressive decrease in their number was, however, observed (Fig. 4), in accordance with previous findings showing apoptosis from E17.5 (Chen et al., 2001). In order to clarify whether the absence of Prrxl1 induces cell death only in neurons that normally express it or, alternatively, also affects the other Prrxl1-negative glutamatergic and the GABAergic subpopulations by following another differentiation pathway, the expression profile of the spinal dorsal horn of P7 *Prrxl1*$^{-/-}$ mice was compared with that of wild-type mice (Fig. 5). This developmental age was selected because Prrxl1 expression markedly decreases after this age (Rebelo et al., 2007), while the amount of Tlx3- and Lmx1b-expressing neurons is similar to that observed at P21.

In the P7 mutant mice, Tlx3- and Lmx1b-positive neurons were highly packed in a narrow strand at the dorsal horn surface (Fig. 5B,E). Notably, the number of Tlx3- or Lmx1b-positive neurons in the *Prrxl1*$^{-/-}$ mice was similar to those of neurons Prrxl1-negative but Tlx3- and Lmx1b-positive (Fig. 5F) in wild-type mice. These data indicate that, in the absence of Prrxl1, there are no additional neurons differentiating into the Prrxl1$^-$/ Tlx3$^+$/Lmx1b$^+$ subpopulation (Supp. Fig. S3), which accounts for about 30% of the entire glutamatergic population of the superficial dorsal horn (Fig. 3E). Similarly, the number of Pax2-positive neurons was identical in wild-type and *Prrxl1*$^{-/-}$ mice (Fig. 5G–I), suggesting that the development of GABAergic neurons in the superficial dorsal horn is not influenced by Prrxl1.

Departing from the dramatic loss of superficial dorsal horn neurons in *Prrxl1*$^{-/-}$ mice, we investigated how such loss affected the anatomy of the spinal cord dorsal horn using the Golgi-Rio Hortega method. The dorsal funiculus and the dorsal horn of *Prrxl1*$^{-/-}$ mice were shorter dorsoventrally as compared to the wild-type (Fig. 6A,B,E), as previously described (Chen et al., 2001). As in other mammals (Beal et al., 1981;

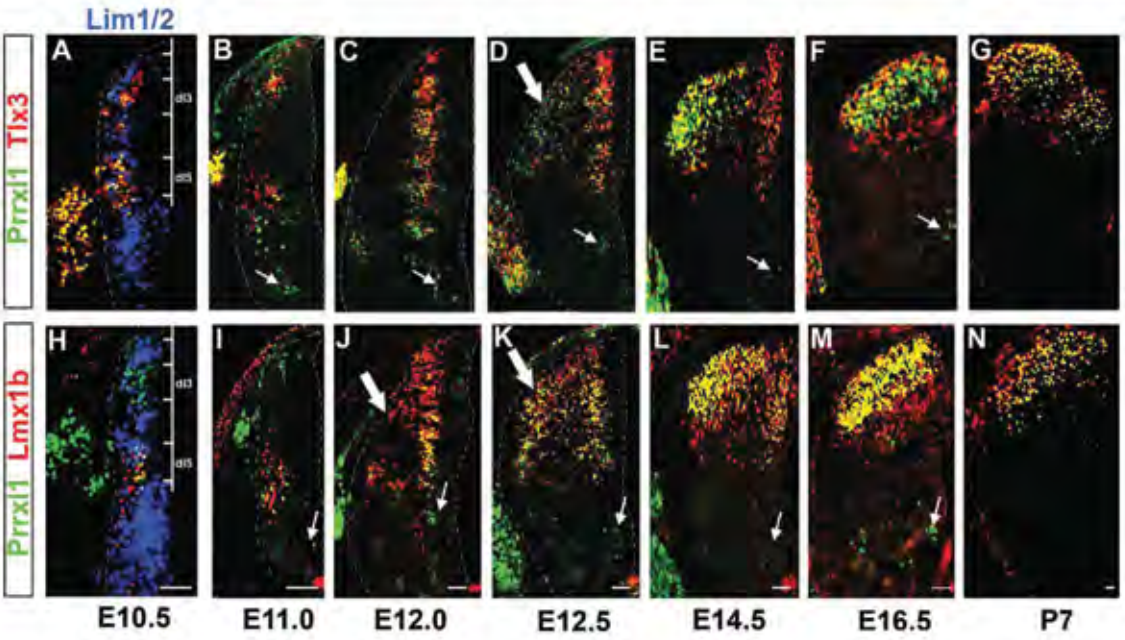

Fig. 1.

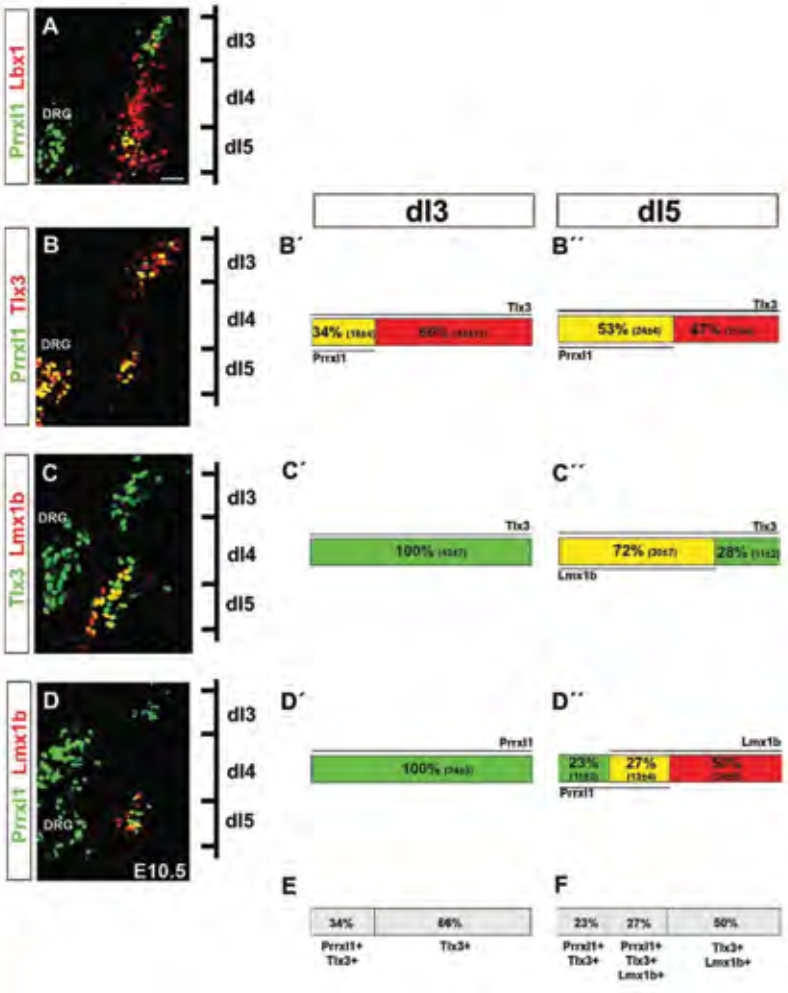

Fig. 2.

Fig. 1. Migration pattern of glutamatergic neurons in the developing spinal cord. Expression of Prrxl1 together with Tlx3 (**A–G**) and Lmx1b (**H–N**) from E10.5 to P7. Lim1/2 was used to better identify the various early-born subpopulations. Small arrows, ventrally migrating dI5 neurons; large arrows, late-born neurons in lateral migration. Dashed white lines demarcate the neural tube boundaries and the ventricular zone. Scale bars = 50 μm.

Fig. 2. Differential co-expression of Prrxl1, Tlx3, and Lmx1b defines subsets of early-born glutamatergic neurons. **A:** Double-immunostaining for Prrxl1 and Lbx1 was used to define dI3 to dI5 neuronal subpopulations. **B–B′:** Double-immunostaining for Prrxl1 and Tlx3 showed that all Prrxl1-positive neurons co-localize Tlx3 in the dI3 and dI5 regions and represent, respectively, 34 and 53% of Tlx3-positive neurons. **C–C″:** Double-immunostaining for Tlx3 and Lmx1b showed that, in the dI5, all Lmx1b-positive cells are also Tlx3-positive and represent 72% of Tlx3-positive neurons. **D–D″:** Double-immunostaining for Prrxl1 and Lmx1b revealed that, in dI5, 27% of stained neurons co-express both transcription factors. **E,F:** Early born neuronal subtypes in dI3 and dI5 regions according to the overlapping expression of Prrxl1, Tlx3, and Lmx1b. Scale bar = 50 μm.

Developmental Dynamics

TABLE 1. Prrxl1 and Tlx3 Co-Expression in Superficial Laminae of P7 Dorsal Horn Spinal Cord

	Nuclei number per section			Co-localization (%)	
Laminae	Prrxl1	Prrxl1+ Tlx3	Tlx3	Prrxl1+ Tlx3/Prrxl1	Prrxl1+ Tlx3/Tlx3
I	62 ± 20	58 ± 18	100 ± 30	93	58
II	224 ± 52	196 ± 44	270 ± 38	88	73
III	82 ± 22	42 ± 22	64 ± 26	51	66
Total	368 ± 92	296 ± 84	434 ± 94	81	68

TABLE 2. Tlx3 and Lmx1b Co-Expression in Superficial Laminae of P7 Dorsal Horn Spinal Cord

	Nuclei number per section			Co-localization (%)	
Laminae	Tlx3	Tlx3 + Lmx1b	Lmx1b	Tlx3+ Lmx1b/Tlx3	Tlx3+ Lmx1b/Lmx1b
I	94 ± 30	94 ± 30	95 ± 30	100	99
II	264 ± 38	264 ± 38	275 ± 70	100	96
III	64 ± 26	64 ± 26	100 ± 34	100	64
Total	422 ± 94	422 ± 94	470 ± 134	100	90

TABLE 3. Prrxl1 and Lmx1b Co-Expression in Superficial Laminae of P7 Dorsal Horn Spinal Cord

	Nuclei number per section			Co-localization (%)	
Laminae	Prrxl1	Prrxl1 + Lmx1b	Lmx1b	Prrxl1+ Lmx1b/Prrxl1	Prrxl1+ Lmx1b/Lmx1b
I	52 ± 24	50 ± 24	94 ± 30	96	53
II	230 ± 54	198 ± 58	278 ± 80	86	71
III	60 ± 24	56 ± 22	94 ± 34	93	60
Total	342 ± 102	304 ± 104	466 ± 144	89	65

Lima and Coimbra, 1986), the most superficial laminae of the spinal dorsal horn of wild-type mice were characterized by the presence of small-size spiny neurons, which amounted to 352. In contrast, the population of the deep dorsal horn (390) included a large amount of neurons with large cell bodies and aspiny dendritic arbors (Fig. 6B–D). In the mutant mice, small neurons at the surface of the dorsal horn were scarce (56), although neurons belonging in the four neuronal types typically present in lamina I (Lima and Coimbra, 1983; Galhardo and Lima, 1999; Galhardo et al., 2000) could be recognized (Fig. 6A,E). Deep dorsal horn neurons were similar in number (422) to the wild-type and were located near the dorsal surface (Fig. 6A,E). These data indicate that, in the absence of Prrxl1, superficial dorsal horn laminae are reduced to a narrow strand and deprived of most of their neurons, while the deep dorsal horn is not affected.

Defective Nociception Activation of the Spinal Cord Dorsal Horn in Prrxl1$^{-/-}$ Mice

Taking into account the marked fall in number of small size neurons in the superficial dorsal horn of *Prrxl1*$^{-/-}$ mice, we investigated to what extent the mutation affected noxious-induced neuronal activation at the spinal level. To address this issue, spinal expression of the Fos protein, a marker of neuronal activity (Hunt et al., 1987; Menétrey et al., 1989), was assessed by immunohistochemistry in wild-type and mutant P21 mice after somatic and visceral noxious stimulation. Counting of Fos-immunoreactive cells was referred to the superficial dorsal horn and the deep dorsal horn. Bearing in mind that the aberrant morphology of the spinal cord of *Prrxl1*$^{-/-}$ mice impaired its correct lamination and that the deep dorsal horn seemed not to be affected in Golgi impregnations, we considered as the deep dorsal horn in the mutant the area equal in ventrodorsal extent from the central canal plane to that defined as the deep dorsal horn in wild-type mice.

No Fos-immunoreactivity was detected in the spinal cord of either wild-type or mutant animals not subject to noxious stimulation. In wild-type mice, neurons Fos-immunoreactive after thermal noxious stimulation were located in the medial third of the superficial dorsal horn (31 ± 9) and in the deep dorsal horn (27 ± 8) (Fig. 7A,C,D). In *Prrxl1*$^{-/-}$ mice, a notable reduction in the number of Fos-expressing neurons occurred in the superficial dorsal horn (5 ± 2), but not in the deep dorsal horn (19 ± 8) (Fig. 7B–D). In wild-type mice, after visceral stimulation, Fos-staining was observed in the superficial dorsal horn (38 ± 6), as well as in the neck of the dorsal horn and in lamina X (79 ± 10) (Fig. 7E,G,H). In mutant mice, the superficial dorsal horn was almost deprived of immunoreactive neurons (9 ± 6), whereas the deep dorsal horn presented a similar amount (58 ± 18) of similarly located Fos-positive neurons (Fig. 7F–H).

The fact that Fos expression in the *Prrxl1*$^{-/-}$ mice was dramatically reduced in the superficial dorsal horn, but not in the deep dorsal horn, confirms the marked impairment of superficial dorsal horn development in the mutant mice.

DISCUSSION

Differential Co-Expression of Prrxl1, Tlx3, and Lmx1b Underlies Cellular Heterogeneity Among Superficial Dorsal Horn Glutamatergic Neurons

Most of the ascending projection neurons and local circuit interneurons present in the dorsal horn of the spinal cord are excitatory and use glutamate as neurotransmitter (Azkue

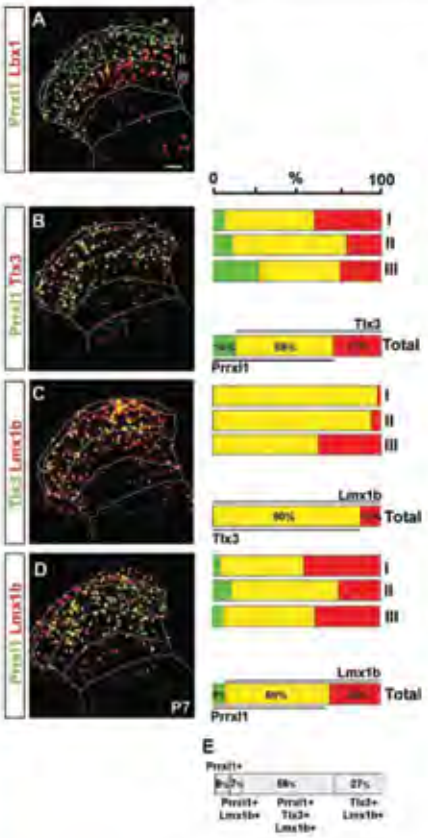

Fig. 3.

Fig. 3. Differential co-expression of Prrxl1, Tlx3, and Lmx1b defines subsets of late-born glutamatergic neurons. **A:** Double-immunostaining for Prrxl1 and Lbx1 showed that Prrxl1-positive neurons co-localize extensively with Lbx1 in lamina III. **B:** Double-immunostaining for Prrxl1 and Tlx3 revealed that 59% of Prrxl1-positive neurons are also Tlx3-positive, and are similarly distributed through lamina I–III. **C:** Double-immunostaining for Tlx3 and Lmx1b showed that Tlx3-positive neurons entirely co-localize Lmx1b, and that neurons expressing only Lmx1b are mostly distributed through lamina III. **D:** Double-immunostaining for Prrxl1 and Lmx1b revealed that most of Prrxl1-positive neurons co-localize Lmx1b, amounting to 60% of all stained neurons. **E:** Late-born glutamatergic neuronal subtypes according to the overlapping expression of Prrxl1, Tlx3, and Lmx1b. Charts were designed using values from Tables 1–3. Scale bar = 50 μm.

et al., 1998; Lu and Perl, 2003; Santos et al., 2007). Here we show for the first time that the superficial dorsal horn glutamatergic population expressing Tlx3 and/or Lmx1b (Cheng at al., 2004), comprises various subsets of neurons as defined by the differential combinatorial expression with the transcription factor Prrxl1. Postnatally four subpopulations were identified ($Prrxl1^{+}/Tlx3^{-}/Lmx1b^{-}$, $Prrxl1^{+}/Tlx3^{+}/Lmx1b^{-}$, $Prrxl1^{+}/Tlx3^{-}/Lmx1b^{+}$, and $Prrxl1^{-}/Tlx3^{+}/Lmx1b^{+}$), while early-born neurons were distributed through two subpopulations in dI3 ($Prrxl1^{+}/Tlx3^{+}$ and $Prrxl1^{-}/Tlx3^{+}$) and three subpopulations in dI5 ($Prrxl1^{+}/Tlx3^{+}/Lmx1b^{+}$, $Prrxl1^{+}/Tlx3^{+}/Lmx1b^{-}$ and $Prrxl1^{-}/Tlx3^{+}/Lmx1b^{+}$). Curiously, while all newly formed glutamatergic neurons express the glutamatergic fate determinant gene, Tlx3 (Cheng et al., 2004), postnatally two of the four subpopulations described do not express Tlx3 and the great majority of glutamatergic neurons are Lmx1b-positive. This is, however, in accordance with recent findings from Xu and collaborators (2008) showing that some

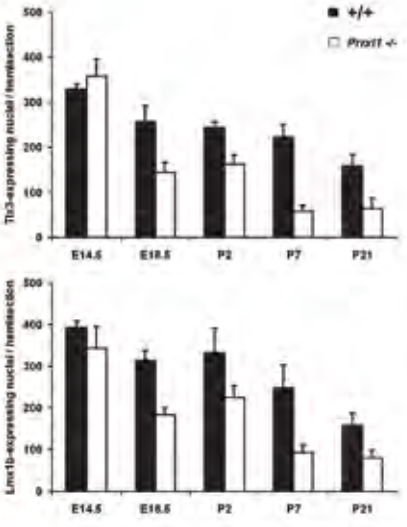

Fig. 4. Loss of Tlx3- and Lmx1b-expressing neurons along development. Tlx3- and Lmx1b-expressing cells were counted in wild-type (close bars) and *Prrxl1*$^{-/-}$ (open bars) spinal cords along development. The reduction in Tlx3- and Lmx1b-positive cells is observed already at E18.5, reaching the lowest amounts at P7.

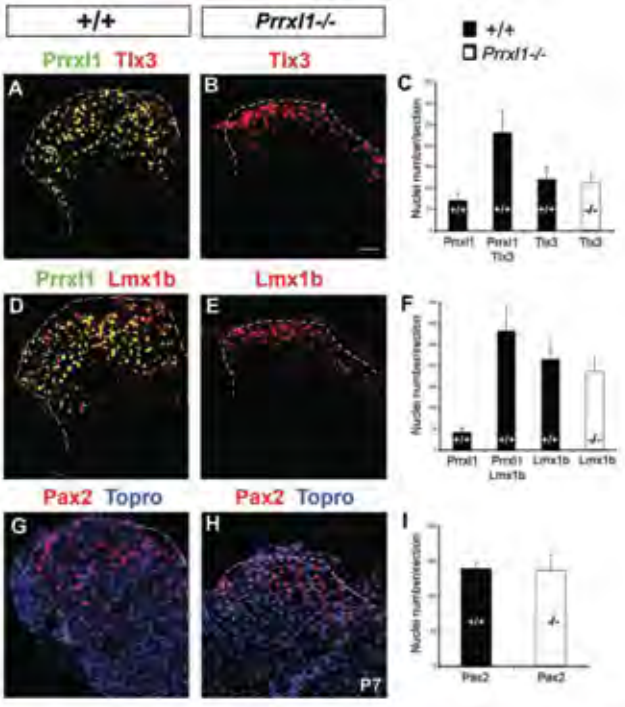

Fig. 5. Glutamatergic superficial dorsal horn neurons in *Prrxl1*$^{-/-}$ mice. **A–C:** Immunostaining for Prrxl1 and Tlx3 showed that in *Prrxl1*$^{-/-}$ mice, Tlx3-positive neurons are concentrated at the dorsal horn surface. Analysis of the number of neurons immunoreactive only for Prrxl1, for Prrxl1 plus Tlx3, and only for Tlx3 in wild-type (closed bars) and *Prrxl1*$^{-/-}$ mice (open bars) revealed that the number of neurons preserved in mutant mice is similar to that of neurons expressing only Tlx3 in the wild-type. **D–F:** Immunostaining for Prrxl1 and Lmx1b revealed that in *Prrxl1*$^{-/-}$ mice, Lmx1b-positive neurons are concentrated at the dorsal horn surface. Analysis of the number of neurons immunoreactive for Prrxl1 and Lmx1b in wild-type (closed bars) and *Prrxl1*$^{-/-}$ mice (open bars) revealed that the number of preserved neurons in mutant mice is similar to that of neurons expressing only Lmx1b in the wild-type. **G–I:** Pax2-stained sections showed a similar amount of GABAergic Pax2-positive neurons both in wild-type and *Prrxl1*$^{-/-}$ mice. Nuclear counterstaining was performed with Topro. Scale bar = 50 μm.

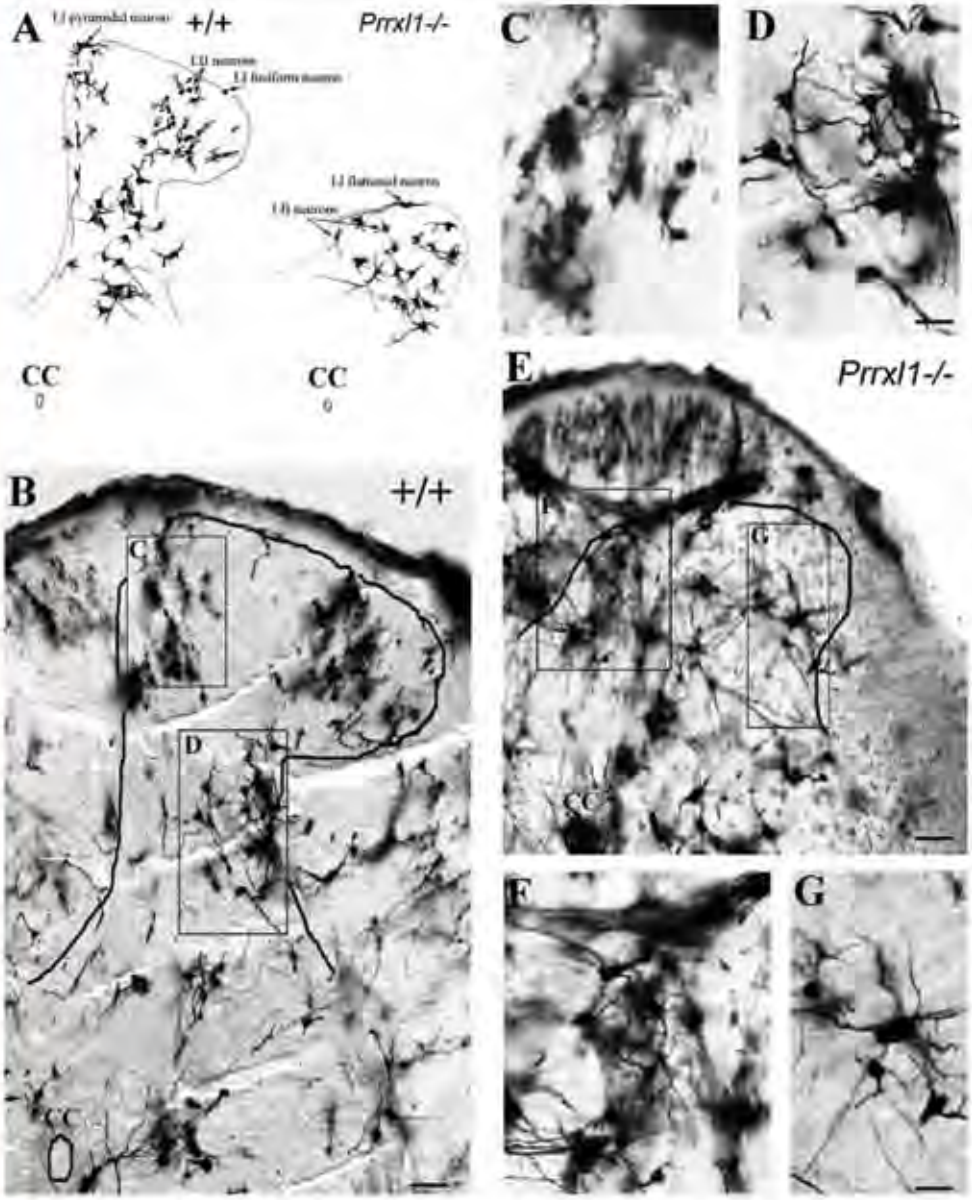

Fig. 6. Superficial dorsal horn neuronal arrangement is compromised in the absence of Prrxl1. **A:** Drawings representing all Golgi-stained neurons in the spinal dorsal horn sections shown in B and E, from, respectively, P7 wild-type (+/+) and *Prrxl1*$^{-/-}$ mice. Examples of typical laminae I and II neurons are shown (arrows). **B–D:** Photomicrographs of Golgi-stained neurons in wild-type mice showing small-size spiny neurons in the superficial dorsal horn (see close-up in C) and large aspiny neurons in the deep dorsal horn (see close-up in D). **E–G:** In *Prrxl1*$^{-/-}$ mice, large-diameter deep dorsal horn neurons extend to near the dorsal surface together with a few small-size neurons (see close-ups in F and G). CC, central canal. Scale bars = 200 μm (B, E) and 100 μm (C, D, F, G).

Tlx3-dependent neurons located in lamina III, in the same location as the Prrxl1^{+}/Tlx3^{-}/Lmx1b^{+} described here, do no longer express Tlx3 postnatally.

In *Prrxl1*$^{-/-}$ spinal cord, both the initial migration pattern and the differentiation of Lmx1b neurons do not seem to be affected until E14.5 (Chen et al., 2001). At E15.5, a decrease in Nissl staining compared to wild type mice is detected, whereas higher levels of apoptosis are observed at E17.5 (Chen et al., 2001). The present observation that, in this mutant, a large fraction of Tlx3-positive and Lmx1b-positive superficial dorsal horn neurons is absent from E18.5 on is in line with these previous findings on putative neuronal degeneration after E15.5 in the absence of Prrxl1 (Chen et al., 2001), and reveals the glutamatergic nature of this Prrxl1-dependent population. In this respect, it should be noted that, although lamina II neurons have for long been claimed to exert an inhibitory role (Willis and Coggeshall, 1991), the occurrence of an excitatory, glutamatergic subpopulation amounting to 85% of lamina II neurons was recently demonstrated (Santos et al., 2007, 2009), in agreement with developmental data showing that Tlx3-positive neurons populate this lamina (Cheng et al., 2004) and the fact that Pax2-positive neurons amount to about 20% of laminae I–III neurons (present results). Moreover, the present study clearly shows that in *Prrxl1*$^{-/-}$ mice, the superficial dorsal horn glutamatergic population that does not express Prrxl1(Prrxl1^{-}/Tlx3^{+}/Lmx1b^{+}) is preserved in amounts similar to those occurring in the wild-type mice. The same occurred with the GABAergic (Pax-2-positive) population, further supporting that Prrxl1-dependent neurons degenerate instead of following another differentiation pathway.

Prrxl1 spinal expression is highly reduced in *Tlx3* knockout mice around E16.5, and completely abolished in *Tlx3*/*Tlx1* double knockouts (Qian et al., 2002). This suggests that the Prrxl1-expressing neurons present in the *Tlx3* mutant mice may

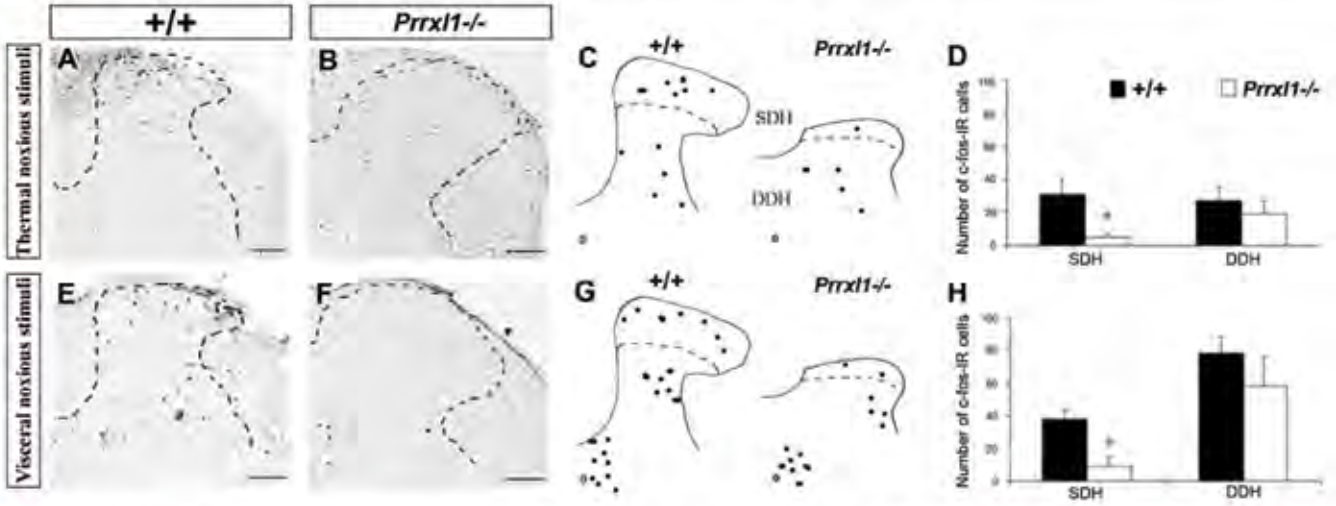

Fig. 7. Spinal c-fos expression after noxious stimulation is altered in the mutant Prrxl1 mice. **A, B, E, F:** Photomicrographs of Fos immunostaining in wild-type and *Prxl1*$^{-/-}$ mice after thermal (A, B) and visceral (E, F) noxious stimulation. **C, G:** Camera lucida drawings of Fos-immunoreactive neurons in section of wild-type and mutant mice after thermal (C) and visceral (G) noxious stimulation; each dot represents 5 Fos-positive neurons. A reduction in the number of Fos-positive neurons in the superficial dorsal horn of mutant mice was observed. **D, H:** Quantitative analysis of the number of Fos-immunoreactive (IR) cells after thermal (D) and visceral (H) noxious stimulation in wild-type (closed bars) and *Prxl1*$^{-/-}$ (open bars) mice revealed a significant reduction in the number of Fos-positive neurons in the superficial dorsal horn (SDH) of mutant mice, but not in the deep dorsal horn (DDH). *$P < 0.05$. Scale bars = 100 μm.

correspond to the Prrxl1^{+}/Tlx3^{-}/Lmx1b^{-} and Prrxl1^{+}/Tlx3^{-}/Lmx1b^{-} subpopulations described here, which are therefore likely to also express Tlx1. On the other hand, in *Lmx1b*$^{-/-}$ mice at E15.5, Prrxl1 expression was reported to be totally absent (Ding et al., 2004). Here, however, we uncovered a minute proportion of both early-born and late-born Prrxl1-positive neurons that does not express Lmx1b. Taking into account that the anti-Prrxl1 antibody used in this study does not discriminate between Prrxl1 and its recently described spliced variant Prrxl1-b (Rebelo et al., 2009), it is possible that the Prrxl1^{+}/Tlx3^{-}/Lmx1b^{-} neurons express the splice variant of Prrxl1b.

Spinal Nociceptive Processing Impairment in the Absence of Prrxl1

In the spinal cord dorsal horn, neurons relaying nociceptive input to supraspinal levels are distributed through lamina I and the deep dorsal horn, while lamina II is almost exclusively populated by local circuit, modulatory interneurons (Willis and Coggeshall, 1991). Here we used the Golgi-Rio Hortega silver impregnation method to better evaluate to which extent the loss of Prrxl1-expressing neurons affected the neuronal population of the superficial and deep dorsal horn laminae. In *Prrxl1*$^{-/-}$ mice, a clear reduction (85%) in the number of superficial dorsal horn neurons was observed, whereas no changes were detected among neurons of the deep dorsal horn. The number of Golgi-impregnated superficial neurons was, however, far below what would be expected from the sum of glutamatergic Prrxl1-independent neurons and GABAergic Pax2-positive neurons at P7 (about 50% of the total dorsal horn neurons) (Fig. 5). The fact that the Golgi method impregnates a small fraction of the neurons present in a certain area may explain these figures, although the possibility that, due to reorganization of the dorsal horn, some of these neurons are now intermingled with deep dorsal horn neurons cannot be ruled out.

The marked decrease in the number of noxious-evoked Fos-positive neurons in the superficial dorsal horn of *Prrxl1*$^{-/-}$ mice was not accompanied by any change in Fos- activation in the deep dorsal horn. This points to preserved peripheral nociceptive innervation of the deep dorsal horn in the absence of Prrxl1. Previous studies have shown that, although in *Prrxl1*$^{-/-}$ mice marked postnatal death of small-size DRG neurons takes place with disruption of sensory innervation of various peripheral tissues, about 2/3 of the peptidergic and non-peptidergic DRG neurons survive (Rebelo et al., 2006). The present finding strongly suggests that these remaining primary afferent neurons are those that innervate the deep dorsal horn, and reinforces the hypothesis that postnatal death of DRG neurons in *Prrxl1*$^{-/-}$ mice might be due to them not finding their neuronal targets in the superficial dorsal horn.

On the other hand, preserved noxious-evoked activation of deep dorsal horn neurons in *Prrxl1*$^{-/-}$ mice suggests that the amputation of the spinal cord superficial modulatory circuitry does not significantly affect nociceptive processing in the deep dorsal horn. According to the molecular characterization of the superficial dorsal horn neuronal population carried out here, the local spinal cord pain control circuit of *Prrxl1*$^{-/-}$ mice must be deprived of an important excitatory component. The absence of lamina II excitatory interneurons should result in substantially reduced activation of deep dorsal horn projecting neurons by disrupting the balance between excitation and inhibition of nociceptive transmission,

namely through local GABAergic neurons, whose numbers were not changed. The unexpected observation of unchanged *c-fos* induction levels in the deep dorsal horn raises the possibility that the spinal and supraspinal pain control system has adapted to this condition during development in order to reestablish the lost balance.

However, the apparently normal activation of nociceptive deep dorsal horn neurons in *Prrxl1*$^{-/-}$ mice (present data) coexists with a significant depression of nociceptive responses in a variety of acute pain behavioral tests (Chen et al., 2001). This poses interesting questions as to the relative role of lamina I and deep dorsal horn transmission neurons in pain processing and reveals that *Prrxl1*$^{-/-}$ mice may constitute a unique model to address this issue, provided that postnatal survival can be prolonged. Anatomofunctional studies of the nociceptive neuronal network in this model would give invaluable information on the role of deep dorsal horn projection neurons and substantia gelatinosa modulatory interneurons in pain processing.

EXPERIMENTAL PROCEDURES

Animal Manipulation

Wild-type mice and *Prrxl1*$^{-/-}$ littermates were generated by heterozygote intercrosses and genotyped as previously described (Chen et al., 2001). Animals were bred and maintained at the IBMC animal facility under temperature- and light-controlled conditions. The embryonic day 0.5 (E0.5) was considered to be the midday of the vaginal plug. Experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) and the ethical guidelines for pain investigation in animals (Zimmermann, 1983).

Tissue Preparation

Embryos were removed by caesarian surgery of pregnant females under sodium pentobarbital anaesthesia (50 mg/kg i.p.), fixed by immersion for 4 hr in freshly prepared 4% paraformaldehyde in 0.1M phosphate-buffered saline pH 7.4 (PBS), cryoprotected in 30% sucrose in PBS overnight, and sectioned transversally at the hind-paw level on a cryostat at 12 μm. Post-natal mice were perfused through the ascending aorta with 5 ml of PBS followed by 50 ml of 4% paraformaldehyde in PBS. Spinal cords were removed, immersed in the same fixative for 2 hr, and cryoprotected in 30% sucrose in PBS overnight. Lumbar coronal frozen sections were cut on a cryostat at 12 μm, except for tissue that underwent *c-fos* analysis or Golgi impregnation, in which case one in every four 40-μm-thick cryostat sections and serial 150-μm frozen sections, respectively, were cut from spinal segments L4 and L5.

Immunohistochemistry

Primary antibodies used were: rabbit anti-Prrxl1 (1:500; Rebelo et al., 2007), guinea pig anti-Tlx3, anti-Lmx1b, and anti-Lbx1 (1:1,000; kindly provided by C. Birchmeier and T. Müller), rabbit anti-Pax2 (1:1,000, Invitrogen), rabbit anti-Fos (1:10,000, Oncogene Science, Gaithersburg, MD), and mouse anti-Lim1/2 (1:20, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Secondary Alexa-conjugated antibodies (Invitrogen, Carlsbad, CA) were used to detect Prrxl1, Tlx3, Lmx1b, Lbx1, and Pax2, and biotinylated swine anti-rabbit antibody to detect the Fos protein (Dako, Carpinteria, CA). Vectastain ABC-HRP standard kit (Vector Labs, Burlingame, CA) was used to detect the Fos signal with the DAB chromogen. Sections were cleared in xylol and mounted in Eukitt®. Spinal cord counter-staining was accomplished using Topro (Invitrogen) and images were captured on a Zeiss (Z1, Thornwood, NY) fluorescence microscope equipped with an ApoTome system.

Golgi Impregnation

Spinal cord blocks from L4–L5 removed from wild-type (n = 2) and *Prrxl1*$^{-/-}$ (n = 2) P7 mice were double impregnated by the Golgi-Río Hortega method (Ramón y Cajal and de Castro, 1933; Sotelo and Palay, 1968). Briefly, blocks were immersed in the potassium dichromate solution for 48 hr (renewed after 24 hr), 0.75% silver nitrate overnight, the potassium dichromate solution for another 24 hr, and the silver nitrate solution overnight. Frozen serial 150-μm-thick transverse sections were obtained, and neuronal counting performed using five sections randomly taken from each animal.

Noxious Stimulation for *c-fos* Induction

Experiments were carried out in 22 P21 mice (12 wild-type and 10 *Prrxl1*$^{-/-}$), which were further subdivided in 4 groups. The first group consisted of 4 wild-type and 4 *Prrxl1*$^{-/-}$ mice receiving cutaneous noxious thermal stimulation. Animals were anaesthetized with sodium pentobarbital and their right hindpaw was submersed for 10 sec in hot water (52°C) every minute, during 20 min. The second group, again 4 wild-type and 4 *Prrxl1*$^{-/-}$ mice, was used for visceral noxious stimulation. Anaesthetized mice were placed on a cotton pad, the bladder was exposed through a low midline abdominal incision, and a 25-gauge needle was inserted in the bladder dome. Bladders were then distended with saline (0.9%) at room temperature during 15 min at a constant pressure of 50 cm of H_20. Free outflow through the urethra allowed fluids to be expelled preventing the occurrence of bladder over distension. A third group (n = 4) was used for controlling *c-fos* induction, and consisted of 2 wild-type and 2 *Prrxl1*$^{-/-}$ animals that were maintained for 2 hr under sodium pentobarbital anaesthesia (50 mg/kg, i.p.) without any further manipulation. These mice were assumed to indicate the level of *c-fos* induction in non-stimulated animals. The last group consisted of 2 wild-type mice that were only subjected to bladder surgery. Two hours after the onset of manipulation, all animals were transcardially perfused as described above.

Cell Countings/Data Analysis

To quantify Prrxl1-, Lmx1b-, and Tlx3-stained neurons, 15–20 sections from lumbar cord of wild-type (n = 3) and *Prrxl1*$^{-/-}$ mice (n = 3) were used. Cell counting was performed with the ImageJ open source software (Rasband, W., NIH, Bethesda, MD).

Fos-immunoreactive cells present in 10 sections randomly taken from each animal were counted. In order to prevent double counting, sections were separated by at least 120 μm. All Fos-immunoreactive neurons were counted irrespective of the staining intensity, and plotted on drawings of the sections with the aid of a camera lucida. The total number of Fos-positive cells was depicted for each section and the mean number of neurons/section in the superficial dorsal horn (SDH, laminae I–III) and deep dorsal horn (DDH, laminae IV–VI and X) was determined. The distance between a line passing through the central canal and the ventral border of lamina III in wild-type animals was used to define the border between the DDH and the SDH in *Prrxl1*$^{-/-}$ mice (based on the data from Golgi impregnations, which indicated that the deep dorsal horn was not affected in *Prrxl1* knockout mice). Results are presented as the mean ± SEM; differences were compared by one-way ANOVA and statistical significance taken at $P < 0.5$.

Numbers of Golgi impregnated neurons are presented as the total number of neurons observed in the superficial and deep dorsal horn of two wild-type and two knockout mice.

Lamination of the spinal cord dorsal horn was based in Nissl-stained sections, as described previously (Rebelo et al., 2007).

ACKNOWLEDGMENTS

The authors thank Qiufu Ma for a critical reading of the manuscript and Thomas Müller and Carmen Birchmeier for gifts of antibodies.

REFERENCES

Azkue JJ, Mateos JM, Elezgarai I, Benítez R, Lázaro E, Streit P, Grandes P. 1998. Glutamate-like immunoreactivity in ascending spinofugal afferents to the rat periaqueductal grey. Brain Res 790:74–81.

Beal JA, Penny JE, Bickmell HR. 1981. Structural diversity of marginal (lamina-I) neurons in the adult monkey (*Macaca-Mulatta*) lumbosacral spinal cord: a Golgi study. J Comp Neurol 202: 237–254.

Bermingham NA, Hassan BA, Wang VY, Fernandez M, Banfi S, Bellen HJ, Fritzsch B, Zoghbi HY. 2001. Proprioceptor pathway development is dependent on MATH1. Neuron 30:411–422.

Caspary T, Anderson KV. 2003. Patterning cell types in the dorsal spinal cord: what the mouse mutants say. Nat Rev Neurosci 4:289–297.

Chen Z-F, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ. 2001. The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. Neuron 31:59–73.

Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, Arata S, Shirasawa S, Bouchard M, Luo P, Chen C-L, Busslinger M, Goulding M, Onimaru H, Ma Q. 2004. *Tlx3* and *Tlx1* are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci 7:510–517.

Cheng L, Samad OA, Xu Y, Mizuguchi R, Luo P, Shirasawa S, Goulding M, Ma Q. 2005. *Lbx1* and *Tlx3* are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat Neurosci 8:1510–1515.

Dai J-X, Hu Z-L, Shi M, Guo C, Ding Y-Q. 2008. Postnatal ontogeny of the transcription factor *Lmx1b* in the mouse central nervous system. J Comp Neurol 509:341–355.

Ding Y-Q, Yin J, Kania A, Zhao ZQ, Johnson RL, Chen Z-F. 2004. *Lmx1b* controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. Development 131: 3693–3703.

Galhardo V, Lima D. 1999. Structural characterization of marginal (lamina I) spinal cord neurons in the cat: a Golgi study. J Comp Neurol 414:315–333.

Galhardo V, Lima D, Necker R. 2000. Spinomedullary pathways in the pigeon (*Columba livia*): differential involvement of lamina I cells. J Comp Neurol 423:631–645.

Gillespie PG, Walker RG. 2001. Molecular basis of mechanosensory transduction. Nature 413:194–202.

Glasgow SM, Henke RM, MacDonald RJ, Wright CVE, Jonhson JE. 2005. Ptf1 determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. Development 132: 5461–5469.

Goulding M, Lanuza G, Sapir T, Narayan S, 2002. The formation of sensorimotor circuits. Curr Opin Neurobiol 12:508–515.

Gowan K, Helms AW, Hunsaker TL, Collisson T, Ebert PJ, Odom R, Johnson JE. 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron 31:219–232.

Gross MK, Dottori M, Goulding M. 2002. *Lbx1* specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34:535–549.

Helms AW, Johnson JE, 2003. Specification of dorsal spinal cord interneurons. Curr Opin Neurobiol 13:42–49.

Helms AW, Battiste J, Henke RM, Nakada Y, Simplicio N, Guillemot F, Johnson JE. 2005. Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. Development 132:2709–2719.

Hunt SP, Pini A, Evan G. 1987. Induction of c-*fos*-like protein in spinal cord neurons following sensory stimulation. Nature 328:632–634.

Jessell TM. 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1: 20–29.

Julius D, Basbaum AI, 2001. Molecular mechanisms of nociception. Nature 413: 203–210.

Lee KJ, Jessell TM. 1999. The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci 22:261–294.

Lee KJ, Mendelsohn M, Jessell TM. 1998. Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev 12:3394–3407.

Lee KJ, Dietrich P, Jessell TM. 2000. Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. Nature 403:734–740.

Liem KF Jr, Tremmi G, Jessell TM. 1997. A role for the roof plate and its resident TGFβ-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91:127–138.

Lima D, Coimbra A, 1983. The neuronal population of the marginal zone (lamina I) of the rat spinal cord. A study based on reconstructions of serially sectioned cells. Anat Embryol (Berl) 167:273–288.

Lima D, Coimbra A. 1986. A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. J Comp Neurol 244:53–71.

Lu Y, Perl ER. 2003. A specific inhibitory pathway between substancia gelatinosa neurons direct C-fiber input. J Neurosci 23:8752–8758.

Matisse M. 2002. A dorsal elaboration in the spinal cord. Neuron 34:491–497.

Menétrey D, Gannon A, Levine JD, Basbaum AI. 1989. Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular and visceral stimulation. J Comp Neurol 285:177–195.

Müller T, Brohmann H, Pierani A, Heppenstall PA, Lewin GR, Jessell TM, Birchmeier C. 2002. The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34: 551–562.

Nornes HO, Carry M. 1978. Neurogenesis in spinal cord of mouse: an autoradiographic analysis. Brain Res 1:1–16.

Pierani A, Moran-Rivard L, Sunshine MJ, Littman DR, Goulding M, Jessell TM. 2001. Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron 29: 367–384.

Qian Y, Shirasawa S, Chen C-L, Cheng L, Ma Q. 2002. Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes *Rnx/Tlx-3* and *Tlx-1*. Genes Dev 16:1220–1233.

Ramón y Cajal S, de Castro F. 1933. Elementos de Técnica Micrográfica del Sistema Nervioso [Microscopic elements of the nervous system]. Madrid: Tipografia Artistica.

Rebelo S, Chen Z-F, Anderson DJ, Lima D. 2006. Involvement of DRG11 in the development of the primary afferent nociceptive system. Mol Cell Neurosci 33:236–246.

Rebelo S, Reguenga C, Osório L, Pereira C, Lopes C, Lima D. 2007. DRG11 immunohistochemical expression during embryonic development in the mouse. Dev Dyn 236:2653–2660.

Rebelo S, Lopes C, Lima D, Reguenga R. 2009. Expression of a Prrxl1 alternative splice variant during the development of the mouse nociceptive system. Int J Dev Biol 53:1089–1095.

Santos SF, Rebelo S, Derkach VA, Safronov BV. 2007. Excitatory interneurons dominate sensory processing in the spinal substantia gelatinosa of rat. J Physiol 581:241–254.

Santos SF, Luz LL, Szucs P, Lima D, Derkach VA, Safronov BV. 2009. Transmission efficacy and plasticity in glutamatergic synapses formed by excitatory interneurons of the substantia gelatinosa in the rat spinal cord. PLos One 4:e8047.

Sotelo C, Palay SL. 1968. The fine structure of the lateral vestibular nucleus in the rat. I. Neurons and neuroglial cells. J Cell Biol 36:151–179.

Willis WD, Coggeshall RE 1991. Sensory mechanisms of the spinal cord. New York: Plenum Press.

Wine-Lee L, Ahn KJ, Richardson RD, Mishina Y, Lyons KM, Crenshaw EB III. 2004. Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. Development 131: 5393–5403.

Xu Y, Lopes C, Qian Y, Liu Y, Cheng L, Goulding M, Turner EE, Lima D, Ma Q. 2008. Tlx1 and Tlx3 coordinate specification of dorsal horn pain-modulatory peptidergic neurons. J Neurosci 28: 4037–4046

Zhou Q, Anderson DJ. 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109:61–73.

Zimmermann M. 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16: 109–110.

Developmental Dynamics

Int. J. Dev. Biol. 53: 1089-1095 (2009)
doi: 10.1387/ijdb.072507sr

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Expression of a *Prrxl1* alternative splice variant during the development of the mouse nociceptive system

SANDRA REBELO, CLÁUDIA LOPES, DEOLINDA LIMA and CARLOS REGUENGA*

Laboratório de Biologia Celular e Molecular, Faculdade de Medicina, IBMC-Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal

ABSTRACT ***Background*** **Gene expression can be differentially regulated by alternatively spliced transcription factors, providing a mechanism for precise control of diverse morphogenetic events. The paired-type homedomain transcription factor Prrxl1 (formerly known as Drg11) was described as a key regulator of the differentiation of the spinal cord neuronal circuit dedicated to the processing of nociceptive information. Here, we report the characterization of a Prrxl1 alternative splice variant that we termed Prrxl1-b.** ***Methods*** **Mouse *Prrxl1* isoform mRNA sequences were obtained by Rapid Amplification cDNA Ends (RACE) analysis. The distribution and the amount of *Prrxl1-b* at different developmental ages were analyzed by *in situ* hybridization and quantitative real-time PCR, and compared with those of *Prrxl1*.** ***Results*** **The amount of *Prrxl1* was higher than that of the *Prrxl1-b* isoform both in the DRG and the spinal cord. Prrxl1-b contains the N-terminal homeodomain but differs from the previously identified Prrxl1 in the C-terminal part due to alternative mRNA processing. This results in the lack of the OAR domain in the Prrxl1-b primary structure. Prrxl1-b is exclusively localized in neurons primarily involved in the processing of the pain somatosensory modality. *Prrxl1-b* presents the same regional distribution pattern as *Prrxl1*, but differs as to the qualitative and quantitative expression profile at distinct developmental ages in the dorsal root ganglion and spinal cord.** ***Conclusion*** **We suggest that the tissue-specific role of the *Prrxl1* gene may be sustained by an accurate balance in the ratio between the amount of Prrxl1 and its OAR-lacking variant, Prrxl1-b, which may be critical during nociceptive circuit development.**

KEY WORDS: *Prrxl1, homeodomain, splice variant, nociception, Drg11*

Homeodomain proteins are a large family of transcription factors that have been described as important regulators of morphogenesis events (Gehring, 1992). These proteins are characterized by a conserved 60 aminoacid DNA-binding structure, known as the homeodomain that forms three alpha-helices (Gehring *et al.*, 1994; Dragan *et al.*, 2006). This helix-turn-helix structure binds cooperatively as homo- and heterodimers to palindromic DNA regulator sequences (Wilson *et al.*, 1993). Among the different classes of homeoproteins categorized so far, an important group with major roles in embryonic development is that of paired class proteins, characterized by the presence of an additional 128-amino-acid DNA binding domain, referred as the paired domain, located upstream of the homeodomain (Treisman *et al.*, 1991). A related category is the paired-like homeodomain proteins that share high similarity with the paired class proteins but contain a sole DNA-binding region. Additional conserved regions located outside the homeodomain define different subsets of paired-type transcription factors. One of these subfamilies is characterized by the presence of a conserved 14 amino acid motif known as OAR domain, initially described in the homeobox genes Otp (Semina *et al.*, 1996) and Aristaless (Gage and Camper, 1997), located in the carboxyl-terminal tail. To date, the function of this domain is not properly understood. However the exclusive and consistent presence of this motif in the C-terminal of a subset of paired-like homeodomain proteins suggests that it has a molecular function directly related to the transcriptional activity of these factors. One member of this OAR containing paired-type homeodomain family

Abbreviations used in this paper: DRG, dorsal root ganglia; OAR, Otp-Aristaless-Rax domain; ORF, open reading frame; Prrxl1, paired related homeobox protein-like 1.

***Address correspondence to:** Dr. Carlos Reguenga. Instituto de Histologia e Embriologia, Faculdade de Medicina do Porto, Alameda Hernani Monteiro, 4200-319 Porto, Portugal. Tel: +351-22551-3654. Fax: +351-22551-3655. e-mail: cregueng@med.up.pt

Accepted: 13 August 2008. *Published online:* 22 May 2009.

ISSN: Online 1696-3547, Print 0214-6282

is the transcription factor *Prrx1* (official name according to the Mouse Genome Database nomenclature (Eppig *et al.*, 2005)), also referred as *Drg11*.

Analysis of Prrxl1 mutant mice revealed a critical role of this protein in the development of the spinal cord dorsal horn neuronal circuit dedicated to the processing of nociceptive information. *Prrx1*$^{-/-}$ mice exhibit diminished nociceptive behaviour in several pain tests (Chen *et al.*, 2001). Moreover, the differentiation of superficial spinal cord layers is impaired, resulting in structural and neurochemical spinal defects (Chen *et al.*, 2001) and a marked reduction of the number of small primary afferent neurons (Rebelo *et al.*, 2006a).

Here, we report the characterization of a Prrxl1 alternative splice variant that we termed Prrxl1-b. This isoform differs from Prrxl1 in the C-terminal part. The resulting protein contains the N-terminal homeodomain but lacks the C-terminal OAR domain. Moreover, the distribution and the amount of Prrxl1-b at different developmental ages were compared with those of Prrxl1.

Identification of Prrxl1-b and splicing organization of the *Prrxl1* gene

Recently we have characterized the Prrxl1 expression by western-blotting analyses using spinal cord extracts at different developmental ages (Rebelo *et al.*, 2007). Distinct bands were detected suggesting the existence of Prrxl1 isoforms. This observation led us to perform an extensive homology search in GenBank database using the previously known rat Prrxl1 cDNA sequence (accession number NM_145767) (Saito *et al.*, 1995). From this analysis, a murine Prrxl1 related sequence (accession number BC145917) was identified. This cDNA sequence presents an Open Reading Frame (ORF) distinct from that previously described in the rat (Saito *et al.*, 1995), as observed by the nucleotide sequence alignment in Fig. 1A, suggesting the existence of a Prrxl1 variant, which we termed Prrxl1-b. Although the Prrxl1 role has been analyzed in the mouse (Chen *et al.*, 2001; Rebelo *et al.*, 2006a), the murine sequence was not determined so far. We looked for the murine homologous of the previously known rat Prrxl1 using the genomic information of the contig NT-039606. The predicted mouse Prrxl1 ORF sequence was assembled (Fig. 1A). To validate the existence of the two Prrxl1 isoforms, we searched for the respective full-length mRNA. We isolated the 5' and 3' untranslated regions (UTR) by Rapid Amplification cDNA ends (RACE) analyses using primers that hybridize in a region of the open reading frame restricted to each isoform. Only one band corresponding to the 3'UTR of each isoform was obtained (marked by asterisks in Fig. 1B). On the contrary, several sequences with 5'UTR regions of different size were identified suggesting the presence of multiple Prrxl1 transcription start points in the spinal cord (Bands 1 to 3 in Fig. 1B). The sequence of each transcript is presented in Fig. 1C. Note that band 2 in the Prrxl1 5'-RACE reaction contains two different transcripts of same size. From all the obtained transcripts we selected the transcript containing the longer 5'UTR in order to assemble the cDNA sequences of both isoforms (Fig. 2B). The Prrxl1 and Prrxl1-b nucleotide sequences have been submitted to GenBank and have been assigned the accession number EU670677 and EU670678, respectively. BLAST searches in the mouse EST (Expressed Sequenced Tag) database allowed the identification of an incomplete Prrxl1 cDNA sequence (accession number BY729985) derived from DRG extracts, starting at the same nucleotide as those identified in our 5'-RACE analyses. This observation suggests that the Prrxl1 and Prrxl1-b mRNA sequences identified may correspond to the main transcripts in the diverse types of Prrxl1-expressing neurons.

Based on the information of the contig NT-039606, the alignment between the cDNA of the two isoforms and genomic sequences was performed in order to define the splicing organization of the Prrxl1 gene (Fig. 2A). The murine Prrxl1 gene is located on Chromosome 14 spanning approximately 47 kb and gave rise, by alternative processing of the last exon, to two transcripts: Prrxl1 (the murine homologous to the previously known rat Prrxl1) and the spliced variant Prrxl1-b. The two mRNAs encode proteins that have the first 175 amino acids in common due to exons 1-6 which encode the N-terminal paired-type homeodomain (Fig. 2C). The C-terminal OAR domain present in Prrxl1 amino acid sequence is missing in Prrxl1-b due to the substitution of the exon 7 by two exons specific to Prrxl1-b sequence (Fig. 2 B,C). Prrxl1-b contains 220 aminoacids, making this protein approximately 5 kDa shorter than Prrxl1.

Expression of Prrxl1 isoforms

The Prrxl1 expression pattern has been previously defined by *in situ* hybridization (Chen *et al.*, 2001; Qian *et al.*, 2002; Ding *et al.*, 2003) and immunohistochemistry (Rebelo *et al.*, 2007). However, the probe and the antibody used in these studies did not discriminate between Prrxl1 and the spliced variant Prrxl1-b. In order to define the regional distribution of Prrxl1-b, as compared to that of Prrxl1, *in situ* hybridization studies were performed in embryonic day (E) 15.5 and E18.5 embryos using alternatively spliced exon 7-specific riboprobes (Fig. 3). As previously described for Prrxl1 (Chen *et al.*, 2001; Qian *et al.*, 2002; Ding *et al.*, 2003; Rebelo *et al.*, 2007), both Prrxl1 and Prrxl1-b mRNA were expressed in the trigeminal, facial and glossopharyngeal ganglia (Fig. 3 A,C,G,E), the dorsal root ganglia (DRG) (Fig. 3 D,H), the trigeminal spinal nucleus (Fig. 3 B,F) and the spinal cord dorsal horn (Fig. 3 D,H). As expected, all the structures expressing Prrxl1-b are involved in the processing of nociceptive information. Although no obvious significant differences in the localization of the two isoforms were detected, there appeared to be a dramatic reduction in the expression of Prrxl1-b in the spinal cord at E18.5, as compared to the expression of Prrxl1 mRNA at the same age. This could be due to the fact that the Prrxl1-b mRNA expressing levels were not high enough to be detected by the riboprobe. No such difference was observed in the DRG.

To further confirm the presence of Prrxl1-b expression in the E18.5 embryo spinal cord, Reverse Transcriptase-PCR analyses were performed using primers that enable the amplification of a sequence specific to each isoform. Total RNA extracts were obtained from various tissues of E18.5 embryos (Fig. 4). As expected both transcripts were only observed in nervous tissues. Limited expression of Prrxl1 was detected in the brain, which may have been due to the use of whole brain extracts since the expression of this transcription factor is restricted to a few regions of the brainstem (Qian *et al.*, 2002; Ding *et al.*, 2003; Rebelo *et al.*, 2007). As to the DRG at this stage of development, both isoforms

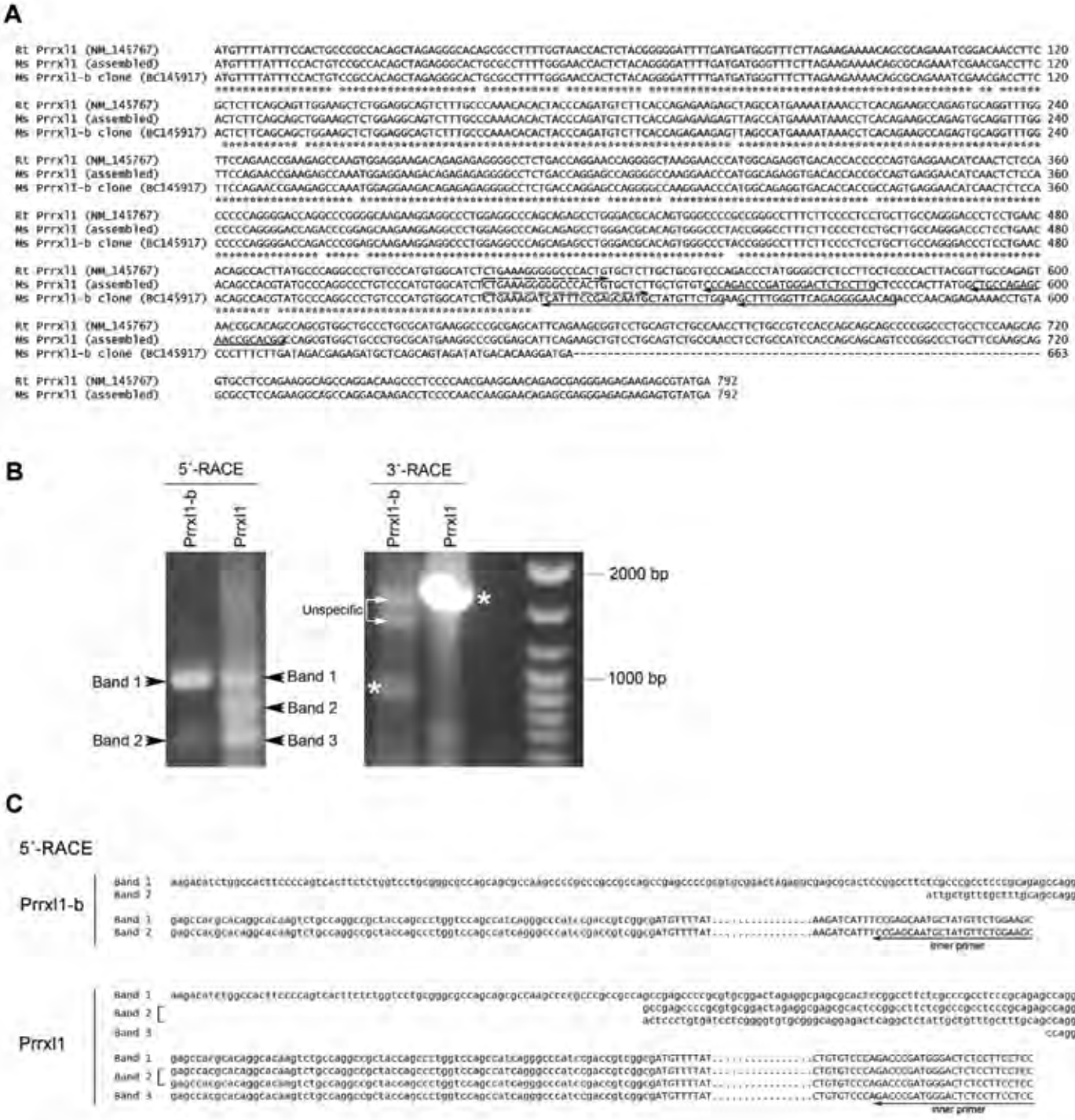

Fig. 1. Identification of *Prrxl1* and *Prrxl1-b* transcripts. (A) *Nucleotide sequence alignment of the Open Reading Frame regions corresponding to the previously known rat* Prrxl1 *(NM_145767), the murine* Prrxl1 *(assembled by comparison with the genomic data in contig NT-039606) and the murine* Prrxl1-b *clone (BC145917). The primers used for 5′ and 3′-RACE reactions are presented.* **(B)** *Gel electrophoresis analysis of the 5′ and 3′-RACE reactions. The asterisks and the arrowheads mark the* Prrxl1 *and* Prrxl1-b *specific bands in, respectively, the 3′ and 5′- RACE reactions.* **(C)** *Nucleotide sequences corresponding to the multiple transcripts obtained in the 5′-RACE analysis. The 5′-UTR nucleotides are in lowercase while the ORF nucleotides are in uppercase.*

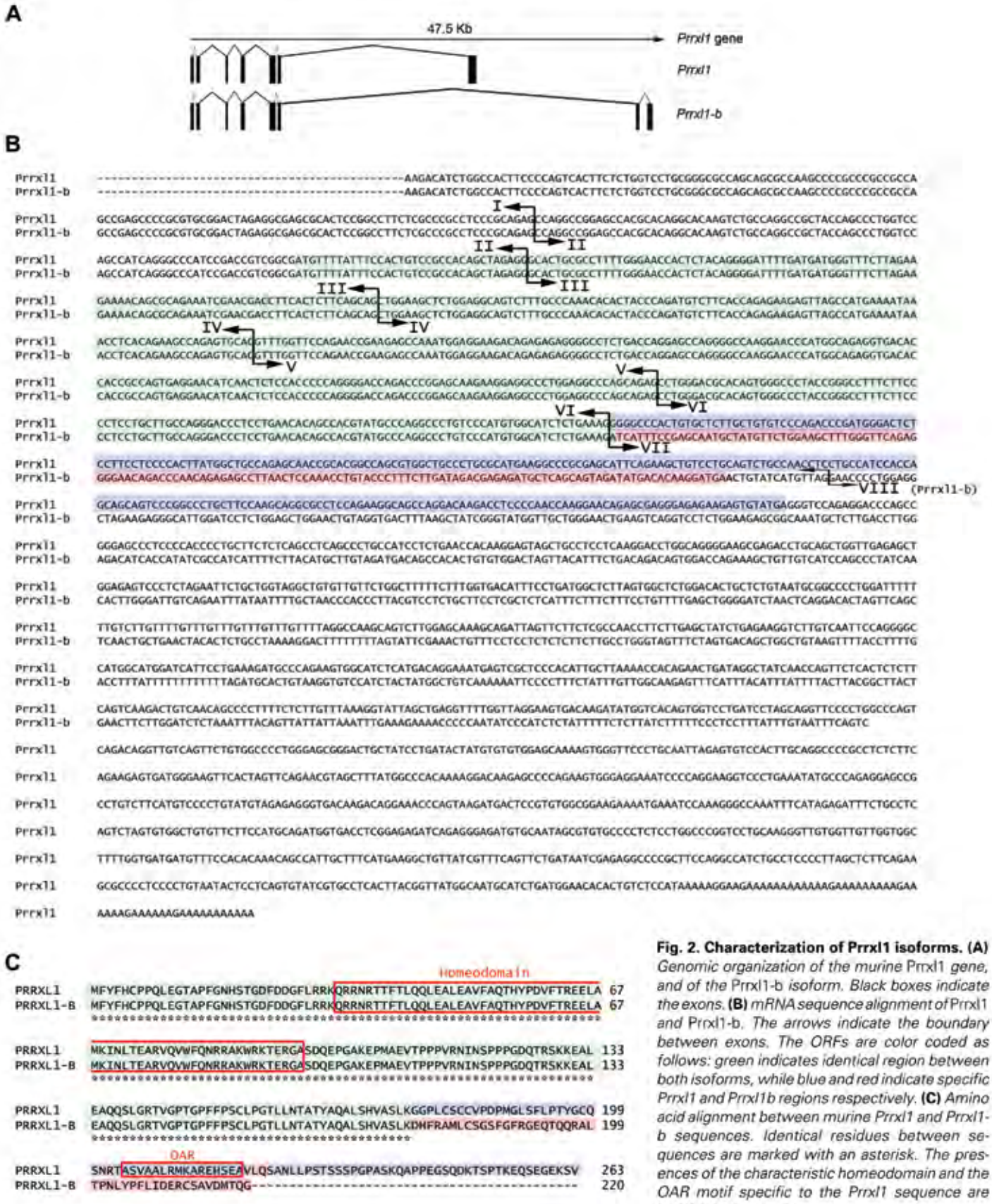

Fig. 2. Characterization of Prrxl1 isoforms. (A) *Genomic organization of the murine* Prrxl1 *gene, and of the* Prrxl1-b *isoform. Black boxes indicate the exons.* **(B)** *mRNA sequence alignment of* Prrxl1 *and* Prrxl1-b. *The arrows indicate the boundary between exons. The ORFs are color coded as follows: green indicates identical region between both isoforms, while blue and red indicate specific Prrxl1 and Prrxl1b regions respectively.* **(C)** *Amino acid alignment between murine Prrxl1 and Prrxl1-b sequences. Identical residues between sequences are marked with an asterisk. The presences of the characteristic homeodomain and the OAR motif specific to the Prrxl1 sequence are highlighted with a box.*

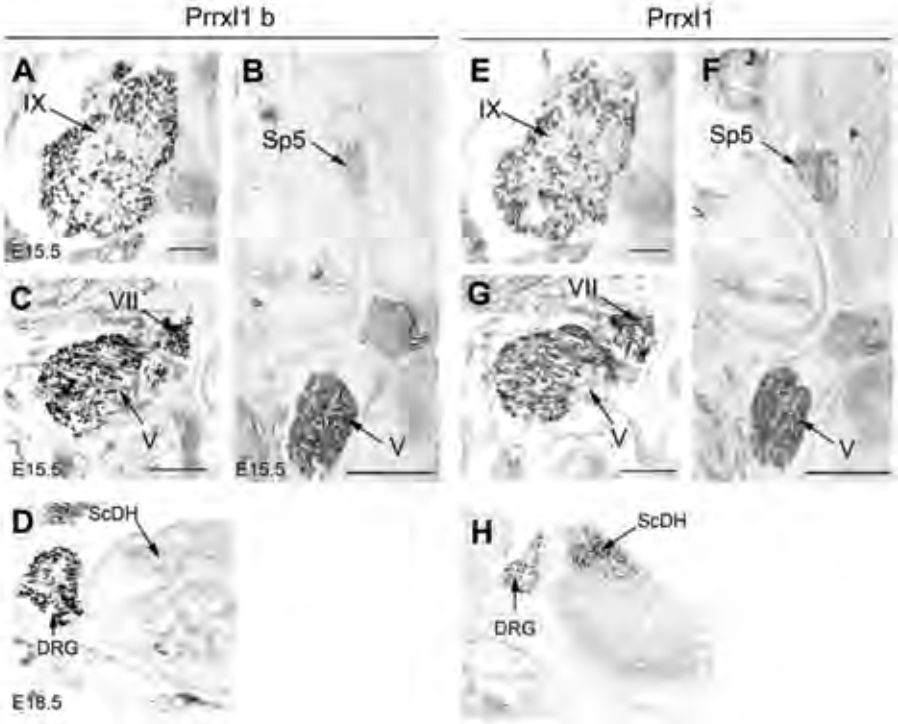

Fig. 3. Distribution of the two *Prrxl1* isoforms. *Comparative expression of* Prrxl1 *and* Prrxl1-b *analysed by in situ hybridization in adjacent cranial transverse sections of E15.5 embryos* **(A,B,C,E,F,G)** *and in spinal sections of E18.5 embryos* **(D,H)**. *V, Trigeminal ganglion; VII, Facial ganglion; IX, Glossopharingeal ganglion; Sp5, Trigeminal spinal nucleus; ScDH, Spinal cord Dorsal Horn; DRG, Dorsal root ganglion. Scale bars: 100 µm in (A,E); 500 µm in (B,C,G,F).*

exhibited high equivalent levels of expression, whereas in the spinal cord Prrxl1-b was far less expressed than Prrxl1. These observations led us to hypothesize that Prrxl1 and Prrxl1-b could be differentially regulated in the spinal cord and DRG.

Differential temporal isoform expression along development

To further characterize putative differences in the expression pattern of Prrxl1 and Prrxl1-b, the amount of each transcript was quantified by real-time PCR analyses at different developmental ages. Although Prrxl1 expression has been previously described to be first detected at E10.5 (Rebelo *et al.*, 2006b; 2007), this analysis started at E15.5 for spinal cord extracts and at E18.5 for DRG extracts due to difficulties in accurately dissecting these tissues at earlier ages. The time-course of expression of Prrxl1 and Prrxl1-b transcript is shown in Fig. 5A. In all the ages analyzed, the amount of Prrxl1 was higher than that of Prrxl1-b both in the DRG and the spinal cord. In the DRG, both transcripts presented the same temporal expression profile: a marked decrease of expression between E18.5 and P0 followed by an increase at P14, coming back to low levels at P21. The ratio between the two transcripts was maintained at similar values from prenatal to postnatal ages (Fig. 5B). In the spinal cord, the level of Prrxl1 mRNA declined progressively from E15.5 to P14, and then decreased abruptly to levels similar to those of Prrxl1-b (Fig. 5A). This pattern was previously reported by western-blotting analyses (Rebelo *et al.*, 2007), suggesting that the mRNA expression correlates with the protein expression. Prrxl1-b was expressed in high amounts at E15.5, but markedly decreased at E18.5 and maintains such low levels from then on (Fig. 5A). In contrast to what was observed in the DRG, the ratio between Prrxl1 and Prrxl1-b in the spinal cord was high between E18.5 and postnatal day (P) 7, reaching a maximum value at P0, and diminished progressively until P21 (Fig. 5B).

Considering the differences verified in the expression profile of the two Prrxl1 isoforms in the spinal cord (Fig. 5), we hypothesized that the precise control of the amount of Prrxl1 relative to Prrxl1-b is a key element in the regulation of the molecular mechanisms that govern the establishment of accurate sensory circuits. The importance of accurate regulation of the amount of this type of paired-like homeodomain transcription factors during development has been previously demonstrated by overexpression of an OAR truncated form of Cart1, which resulted in severe cranial and vertebral malformations due to an increase in DNA binding activity (Brouwer *et al.*, 2003). We therefore suggest the existence of a delicate balance between Prrxl1 and its OAR-lacking variant, Prrxl1-b, which is critical during the nociceptive circuit development. It is known that at E15.5, an age at which the laminar architecture of the murine spinal cord begins to be perceptible, sensory axons projecting to both the superficial and deep dorsal horn have developed exhibiting a distinctive appropriate trajectory towards their central targets (Ozaki and Snider, 1997). In *Prrxl1*$^{-/-}$ mice, the differentiation of superficial spinal cord layers is impaired (Chen *et al.*, 2001), while no obvious alterations are detected in the embryonic development of DRG neurons at this age (Chen *et al.*, 2001; Rebelo *et al.*, 2006a). A significant reduction of the number of small primary afferent neurons was

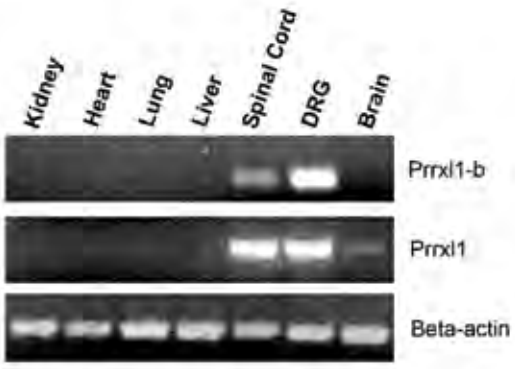

Fig. 4. Pattern of expression of the two *Prrxl1* isoforms. *Tissue specific expression of the* Prrxl1 *and* Prrxl1-b *analysed by reverse-transcriptase PCR in E18.5 embryo. DRG, dorsal root ganglion.*

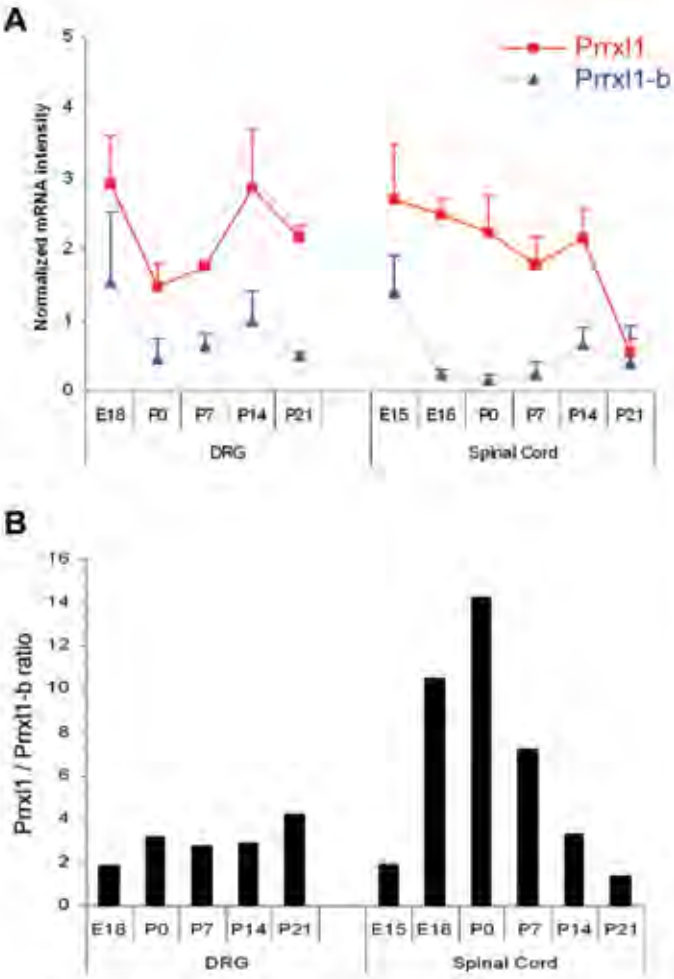

Fig. 5. Relative quantification of *Prrxl1* isoform mRNA determined by real-time PCR during DRG and spinal cord development. (A) *The relative mRNA intensities after normalization with beta-actin at each developmental stage are represented.* **(B)** *Bars represent the ratio between* Prrxl1 *and* Prrxl1-b *mRNA intensity at the various time points.*

only reported at postnatal ages (Rebelo *et al.*, 2006a). These observations suggest that the *Prrxl1* gene governs neuronal differentiation in the spinal cord and neuron survival/maintenance in the DRG. Considering the differences verified in the expression profile of the two Prrxl1 isoforms in the DRG compared to the spinal cord (Fig. 5), we hypothesize that the tissue- specific role of this gene may be sustained by accurate variations in the ratio between the amount of Prrxl1 and Prrxl1-b.

Recently, two isoforms of the Prx1 gene, another paired-like homeodomain transcription factor, have been described as opposing regulators of chondrogenesis (Peterson *et al.*, 2005). Prx1a contains a C-terminal OAR domain while Prx1b lacks the OAR domain due to an alternatively spliced exon 4 (Norris and Kern, 2001a). The function of the conserved OAR motif is still imprecise, but deletion of this domain in the Prx1 protein leads to an increase in DNA binding and transactivation potential (Norris and Kern, 2001b). Since Prrxl1 isoforms are structurally similar to Prx1 isoforms, it is possible that Prrxl1 and Prrxl1-b are also involved in differential transcriptional activity assigned by the occurrence or not of the C-terminal OAR motif. Taking into consideration the significant Prrxl1-b down-regulation observed in the spinal cord between E18.5 and P7, a time interval at which synaptic connectivity is in course (Fitzgerald, 2005), it is tempting to suggest that Prrxl1-b could be acting until that time point as a repressor of synaptic establishment at the superficial dorsal horn, modulating the Prrxl1 transcriptional activity on RGM-B, an axon guidance molecule that has been identified as a target of Prrxl1 (Samad *et al.*, 2004). The generation of an isoform-specific Prrxl1-b gene-targeted mouse is being carried out and will surely help in dissecting the specific function of this paired-like homeodomain transcription factor.

Materials & Methods

In situ *hybridization*

Appropriate stage embryos, determined according to the plug date (considered to be E0.5), or dissected tissues from postnatal mice were fixed overnight with 4% paraformaldehyde buffered with 0.1 M sodium phosphate pH 7.4, cryoprotected with 30% sucrose, embedded in OCT compound (Sakura) and cryosectionned on 12 µm sections. *In situ* hybridization was performed to recognize Prrxl1 and Prrxl1-b mRNA following the procedures described elsewhere (Chen *et al.*, 2001). The following primers were designed on the basis of the alternative exon 7 sequences:

Prrxl1

5'-cccatgtggcatctctgaaag-3'and

5'-tcatacactcttctctccctcgc-3';

Prrxl1-b

5'-cccatgtggcatctctgaaag-3' and

5'-tcatccttgtgtcatatctactgc-3'.

The corresponding DNA fragments were amplified by Reverse-Transcriptase PCR using E18.5 spinal cord total RNA cloned into the pCR4-TOPO plasmid (Invitrogen) and sequenced to confirm the authenticity of the amplicon. After linearization, the recombinant plasmids were used as a template for the *in vitro* RNA transcription of digoxigenin-labeled antisense probes.

Reverse-transcriptase and real-time PCR

Total RNA from tissues of appropriate stage embryos was isolated using the Micro-to-midi total RNA purification System (Invitrogen) following the manufacturer's instructions, analyzed by typical agarose gel electrophoresis to check the RNA integrity and quantified by spectrophotometry. The first strand cDNA synthesis was prepared at 50°C during 1h from 2 µg of total RNA using 200U of Superscript III Reverse Transcriptase enzyme (Invitrogen) and 500 ng of oligo$(dT)_{12-18}$ (Invitrogen). The presence of Prrxl1 and its splice variant Prrxl1-b was revealed by PCR from 2 µl of the first strand cDNA using the primer pairs referred above. The amount of cDNA prepared from each experimental group was normalized with an internal control by amplification of beta-actin using the primer pair 5'-tcatgaagtgtgacgttgacatcc-3' and 5'-gtaaaacgcagctcagtaacagtc-3'. The PCR conditions were the following: denaturation at 94°C for 30 s, annealing at 58°C for 45 s and elongation at 72°C for 45 s during twenty-eight cycles for Prrxl1 and thirty cycles for Prrxl1-b. Equal amounts of the PCR products were subjected to a 2 % agarose gel electrophoresis and visualized by ethidium bromide staining under UV light source. To assess for potential residual genomic DNA in the RNA extracts, a control containing all reagents except the reverse transcriptase enzyme was included for each sample (No RT control).

For the real-time PCR quantification, the 20 µL reaction included 10 µL of IQ Supermix (Bio-Rad), 400 nM of each primer pair and 2 µL of cDNA samples. RT-qPCR was performed on a iCycler iQ realtime thermocycler (Bio-Rad) using the following parameters: an initial denaturation step of 3 min at 94°C, 40 cycles at 94°C for 30 s, 58°C for 30 s and 68°C for 30 s. Specificity of each reaction was ascertained by melting curve analysis, which began at 50°C and increased to 94°C in 1°C increments, and by

agarose gel electrophoresis of the final products. To evaluate the relative PCR efficiencies of both Prrxl1 and Prrxl1-b primers, serial dilutions of first strand product were used to construct standard curves for each gene measurement. Each reaction condition was performed in duplicate and the mean values were used for calculations of mRNA expression. β-actin was used as control to confirm that similar amounts of starting cDNA were used for all stages tested. Minus Reverse Transcriptase and no RNA controls gave similar high threshold cycle values (>37 cycles), demonstrating that contamination did not interfere with the quantified product. Relative fold expression and standard deviation values were calculated as per Livak and Schmittgen (2001). The results are shown as the mean ± SE of three separate quantitative PCR from at least two independent RNA extractions.

Rapid amplification of cDNA ends (RACE)

5′ and 3′-RACE reactions were performed using the FirstChoice RLM-RACE kit (Ambion) with 1 μg of total RNA extracted from mouse E15.5 spinal cord embryo as the starting material, following the instruction manual. For the 5′-RACE, the primers

Prrxl1

5′-ccgtgcggttgctctggcag-3′ (outer) and

5′-gaaggagagtcccatcgggtctgg-3′ (inner) and

Prrxl1-b

5′-ctgttccctctgaacccaaagc-3′ (outer) and

5′-ccagaacatagcattgctcggaaatg-3′ (inner)

were used in the initial and nested PCR amplifications, respectively. For the 3′-RACE, the primers 5′-ctgaaaggggcccactg-3′ for Prrxl1 and 5′-ctgaaagatcatttccgaatg-3′ for Prrxl1-b were used in a unique amplification reaction. The PCR program used was the following: 94°C for 1 min, 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 3 min and a final extension cycle of 72°C for 10 min. The amplified PCR fragments were analysed by 1.5% agarose gel electrophoresis and the higher base pairs band was extracted, cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

Acknowledgements

This work was funded by the FCT grant POCI/SAU-NEU/59093/2004.

References

BROUWER, A., TEN BERGE, D., WIEGERINCK, R. and MEIJLINK, F. (2003). The OAR/aristaless domain of the homeodomain protein Cart1 has an attenuating role *in vivo*. *Mech Dev.* 120: 241-252.

CHEN, Z-F., REBELO, S., WHITE, F., MALMBERG, A.B., BABA, H., LIMA, D., WOOLF, C.J., BASBAUM, A.I. and ANDERSON, D.J. (2001). The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. *Neuron.* 31: 59-73.

DING, Y.Q., YIN, J., XU, H.M., JACQUIN, M.F. and CHEN, Z-F. (2003). Formation of whisker-related principal sensory nucleus-based lemniscal pathway requires a paired homeodomain transcription factor, Drg11. *J. Neurosci.* 23: 7246-7254.

DRAGAN, A.I., LI, Z., MAKEYEVA, E.N., MILGOTINA, E.I., LIU, Y., CRANE-ROBINSON, C. and PRIVALOV, P.L. (2006). Forces Driving the Binding of Homeodomains to DNA. *Biochemistry.* 45: 141-151.

EPPIG, J.T., BULT, C.J., KADIN, J.A., RICHARDSON, J.E., BLAKE, J.A., and the members of the Mouse Genome Database Group. (2005). The Mouse Genome Database (MGD): from genes to mice—a community resource for mouse biology. *Nucleic Acids Res.* 33: D471-D475.

FITZGERALD, F. (2005). The development of nociceptive circuits. *Nat. Rev. Neurosci.* 6: 507-520.

GAGE, P.J. and CAMPER, S.A. (1997). Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum. Mol. Genet.* 6: 457-464.

GEHRING, W.J., AFFOLTER, M. and BURGLIN, T. (1994). Homeodomain Proteins. *Annual Review of Biochemistry.* 63: 487-526.

GEHRING, W.J. (1992). The homeobox in perspective. *Trends Biochem Sci.* 8: 277-280.

LIVAK, K.J. and SCHMITTGEN, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$(-delta delta c(T)) method. *Methods* 25: 402-408.

NORRIS, R.A. and KERN, M.J. (2001a). The identification of Prx1 transcription regulatory domains provides a mechanism for unequal compensation by the Prx1 and Prx2 loci. *J. Biol. Chem.* 276: 26829-26837.

NORRIS, R.A. and KERN, M.J. (2001b). Identification of domains mediating transcription activation, repression, and inhibition in the paired-related homeobox protein, Prx2. *DNA Cell Biol.* 20: 89–99.

OZAKI, S. and SNIDER, W.D. (1997). Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. *J. Comp. Neurol.* 380: 215-229.

PETERSON, R.E., HOFFMAN, S. and KERN, M.J. (2005). Opposing roles of two isoforms of the Prx1 homeobox gene in chondrogenesis. *Dev. Dyn.* 33: 811-821.

QIAN, Y., SHIRASAWA, S., CHEN, C., CHENG, L. and MA, Q. (2002). Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes *Rnx/Tlx-3* and *Tlx-1*. *Genes Dev.* 16: 1220-1233.

REBELO, S., CHEN, Z.F., ANDERSON, D.J. and LIMA, D. (2006a). Involvement of DRG11 in the development of the primary afferent nociceptive system. *Mol. Cell Neurosci.* 33: 236–246.

REBELO, S., REGUENGA, C., LOPES, C., PEREIRA, C., ANDERSON, D. and LIMA, D. (2006b). Cellular diversity of superficial dorsal horn glutamatergic neurons revealed by differential co-expression of Drg11, Tlx3, and Lmx1b. *Program No. 717.4. Neuroscience Meeting Planner. Atlanta, GA.* Society for Neuroscience.

REBELO, S., REGUENGA, C., OSÓRIO, L., PEREIRA, C., LOPES, C. and LIMA, D. (2007) DRG11 immunohistochemical expression during embryonic development in the mouse. *Dev. Dyn.* 236: 2653-2660.

SEMINA, E.V., REITER, R., LEYSENS, N.J., ALWARD, M., SMALL, K.W., DATSON, N.A., SIEGEL-BARTELT, J., BIERKE-NELSON, D., BITOUN, P., ZABEL, B.U., CAREY, J.C. and MURRAY, J.C. (1996) Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat. Genet.* 14: 392-399.

SAITO, T., GREENWOOD, A., SUN, Q. and ANDERSON, D.J. (1995) Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. *Mol. Cell Neurosci.* 6: 280-292.

SAMAD, T.A., SRINIVASAN, ª, KARCHEWSKI, L.A., JEONG, S.J., CAMPAGNA, J.A., JI, R.R., FABRIZIO, D.A., ZHANG, Y., LIN, H.Y., BELL, E. and WOOLF, C.J. (2004). DRAGON: a member of the repulsive guidance molecule-related family of neuronal- and muscle-expressed membrane proteins is regulated by DRG11 and has neuronal adhesive properties. *J. Neurosci.* 24: 2027-2036.

TREISMAN J, HARRIS E, DESPLAN C. (1991). The paired box encodes a second DNA-binding domain in the paired homeo domain protein. *Genes Dev.* 5: 594-604.

WILSON D, SHENG G, LECUIT T, DOSTATNI N, DESPLAN C. (1993). Cooperative dimerization of paired class homeodomains on DNA. *Genes Dev.* 11: 2120-2134.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

See our recent Special Issue ***Fertilization***, in honor of David L. Garbers and edited by Paul M. Wassarman and Victor D. Vacquier at:
http://www.ijdb.ehu.es/web/contents.php?vol=52&issue=5-6

Function and specificity of Hox genes
David Foronda, Luis F. de Navas, Daniel L. Garaulet and Ernesto Sánchez-Herrero
Int. J. Dev. Biol. (2008) 52: doi: 10.1387/ijdb.072462df

The N-terminus zinc finger domain of Xenopus SIP1 is important for neural induction, but not for suppression of Xbra expression
Kazuhiro R. Nitta, Shuji Takahashi, Yoshikazu Haramoto, Masakazu Fukuda, Kousuke Tanegashima, Yasuko Onuma
and Makoto Asashima
Int. J. Dev. Biol. (2007) 51: 321-325

The expression and alternative splicing of alpha-neurexins during Xenopus development
Zhihong Zeng, Colin R. Sharpe, J. Paul Simons and Dariusz C. Górecki
Int. J. Dev. Biol. (2006) 50: 39-46

Regulation of gene expression by Pax6 in ocular cells: a case of tissue-preferred expression of crystallins in lens
Ales Cvekl, Ying Yang, Bharesh K. Chauhan and Kveta Cveklova
Int. J. Dev. Biol. (2004) 48: 829-844

Isolation and growth factor inducibility of the Xenopus laevis Lmx1b gene
Caroline E Haldin, Sarjit Nijjar, Karine Massé, Mark W Barnett and Elizabeth A Jones
Int. J. Dev. Biol. (2003) 47: 253-262

5 yr ISI Impact Factor (2008) = 3.271

III

DISCUSSION

This thesis uncovers Drg11 as the transcription factor responsible for the establishment of the first relay of the ascending nociceptive pathway. It shows that Drg11 plays a crucial role in the differentiation of various classes of superficial spinal dorsal horn neurons, but appears not to influence the normal embryonic development of small diameter, putative nociceptive DRG neurons. Such a differential role in the development of the first and second order nociceptive neurons is not accounted for by a Drg11 isoform, although the combinatorial expression of Drg11 and its isoform differs between the spinal cord and the DRG along development. Taken together, the results lead to the hypothesis that Drg11 directs the connection between primary afferents and second order nociceptive neurons by promoting the differentiation of the latter and signalling correct targeting for their primary afferent pathway.

Throughout most of the thesis, a genetically modified mouse containing a deletion in the Drg11 gene (*Drg11*$^{-/-}$ or Drg11 knockout) was used.

DRG11 IS INVOLVED IN THE DEVELOPMENT OF THE NOCICEPTIVE SYSTEM

Drg11 is a paired-like homeodomain transcription factor first reported by Saito and collaborators (1995) to be expressed in the DRG and spinal cord dorsal horn, and later shown to be also present at the trigeminal complex (Ding et al., 2003). These findings were, however, based on RT-PCR and *in situ* hybridization approaches, and referred to isolated time points during either embryonic development or postnatal life. In this study, a novel antibody against Drg11 was produced, and its expression along development and postnatal life was characterized at the spinal and supraspinal levels (***Publication I***). Drg11 was shown to be expressed throughout development from as early as E10.5 until shortly after birth in first and second order sensory structures along the entire neuroaxis. At the spinal level, Drg11 was expressed in laminae I-III of the superficial dorsal horn and in small diameter DRG neurons. At the supraspinal level, it was expressed in somatic and visceral sensory ganglia, namely the trigeminal, facial, vestibulocochlear, glossopharyngeal and vagus ganglia, and in the spinal trigeminal nucleus (Sp5) (subnucleus caudalis and oralis), principal trigeminal nucleus (Pr5), nucleus of the solitary tract (NTS) and nucleus prepositus (NP). No Drg11 expression was observed in cranial ganglia exclusively subserving motor function.

Notably, both the location of Drg11 along the brain and the size and neurochemical signature of cranial and DRG primary afferent neurons agreed with the spinal superficial dorsal horn location as to a specific role for Drg11 in the development of the nociceptive system. However, Drg11 was also detected in a trigeminal area devoted to tactile sensation, the Pr5 (our own results; Qian et al., 2002; Ding et al., 2003), while at the spinal level a small fraction of Drg11-immunostained neurons was located in lamina III, the site of termination of Aδ D-hair follicle primary afferents (Light and Perl, 1979b; Willis et al., 2004). Hence, although the major sites of termination of tactile primary afferents did not express Drg11, the possibility of an involvement in the establishment of a particular part of the sensory circuit processing innocuous input should be considered.

In order to investigate whether Drg11 is required for the development of the primary nociceptive circuit, *Drg11*$^{-/-}$ mice were generated and characterized as to DRG and spinal cord morphological and neurochemical abnormalities, and nociceptive function was experimentally evaluated (***Publication II***). *Drg11*$^{-/-}$ mice exhibited a distorted spinal cord dorsal horn, with a reduction in the number of small dark Nissl stained neurons and in CGRP and TrkA immunostaining, and a complete absence of PKCγ staining. Additionally, an abnormal distribution of primary afferent fibers in the superficial dorsal horn was observed, with an apparent lateral-to-medial shift in their distribution. Such an aberrant projection could be taken as indicative of a role for Drg11 in medio-lateral somatotopic organization. However, the loss of PKCγ (***Publication II***), Lmx1b and Tlx3 neurons (***Publication IV***) across the entire mediolateral extent, together with the almost complete amputation of the superficial dorsal horn, better revealed by the Golgi staining (Publication IV), is against this possibility.

As to nociceptive behaviour, *Drg11*$^{-/-}$ mice displayed higher response latencies in the hot plate, tail-flick and paw withdrawal tests for thermal sensitivity, and reduced withdrawal response to mechanical stimulation by von Frey filaments. In addition, they exhibited reduced responses to chemical nociceptive stimulation in both the capsaicin and formalin tests. These results indicate that, in the absence of Drg11, mice present reduced sensitivity to noxious stimuli across a broad range of modalities, including mechano-, thermo-, and chemo-sensitivities. Although all the applied tests deal only with cutaneous nociception, it is very likely that similar nociceptive defects occur for visceral and deep tissue stimulation. Peripheral innervation of *Drg11*$^{-/-}$ mice was shown to be disrupted at postnatal ages in the three types of peripheral tissues (***Publication III***), while noxious evoked induction of the c-fos proto-oncogene was compromised at the superficial dorsal horn after stimulation of the three peripheral areas (***Publication IV***).

Sensorimotor functions, mediated by muscle afferent sensory neurons innervating spindle fibers and Golgi tendon organs, were intact in the knockout mice, indicating that Drg11 is not required for the development of the proprioceptive system (***Publication II***). This is consistent with the observation that no evident neuronal loss, morphological defects or abnormal central projections of IA muscle afferent fibers took place in the ventral spinal cord of these mice. It should be noted that disruption of proprioceptive functioning, with impaired hindlimb locomotion, is actually observed in mice bearing mutations in genes required for proprioceptive sensory neuron development or survival (Ernfords et al., 1994; Fariñas et al., 1994; Klein et al., 1994; Arber et al., 2000).

DRG11 APPEARS NOT TO BE INVOLVED IN THE DIFFERENTIATION OF NOCICEPTIVE PRIMARY AFFERENT NEURONS BUT IS REQUIRED FOR THEIR POSTNATAL SURVIVAL

Mouse embryos deficient in Drg11 exhibited abnormalities in the timing and position of the initial ingrowth of primary afferent fiber projections to the spinal cord dorsal horn. Primary afferent fibers approached the spinal cord and entered the dorsal horn gray matter with a delay of 3 to 4 days, to then penetrate biased towards its medial region (***Publication II***). Nevertheless, the development of small size TrkA-positive, CGRP-positive (peptidergic) and IB4-positive (non-peptidergic) (***Publication III***) neurons was not affected until birth, ruling out a role for Drg11 in their differentiation. The data collected in ***publication III*** showed that, until neonatal age, the total numbers of sensory neurons did not differ, nor did the expression of markers for different primary afferent neuronal subtypes between wild-type and *Drg11*$^{-/-}$ mutant mice. Moreover, innervation of peripheral targets was preserved in *Drg11*$^{-/-}$ mice at P0, indicating that peripheral innervation can reach normal development. Consistent with these results was the absence of TUNEL-positive cells in the DRG of *Drg11*$^{-/-}$ mice during embryonic development and at the neonatal stage (***Publications II and III***). Drg11 thus seems to be neither required for the generation, differentiation and survival of primary afferent neurons until birth, nor for the normal innervation of the various peripheral tissues at this time point.

From P7 on (***Publication III***), there was a decrease to about half of the numbers of both peptidergic and non-peptidergic small-diameter primary afferent neurons in *Drg11*$^{-/-}$ mice. Accordingly, size-frequency distribution of DRG neurons led to the conclusion that neurons in the Aβ range were present in numbers similar to those occurring in wild type mice. However, it also revealed that small diameter neurons at the C fiber range were more affected than neurons at the Aδ fiber range. This finding, together with the fact that IB4 neurons comprise mostly C primary afferents (Zwick et al., 2002), implies that the larger fraction of the small diameter neurons preserved in the absence of Drg11 is Aδ peptidergic (CGRP). C and Aδ fiber neurons are known to convey nociceptive and innocuous thermal input from the periphery (McCarthy and Lawson, 1990; Fundin et al., 1997). Yet, innocuous thermal neurons belong mostly in the C unmyelinated group (for review, see

Willis and Coggeshall, 1991). Thus, being mostly Aδ, the non Drg11-dependent small size primary afferent neurons must mainly convey nociceptive input. Taking into account that the deep dorsal horn keeps its normal morphology in *Drg11*$^{-/-}$ mice whereas the superficial dorsal horn almost completely disappears (***Publication IV***), these neurons most likely make up the nociceptive innervation of lamina V (Light and Perl, 1979a,b; Cervero and Connell, 1984).
It should be noted that, in the knockout mice, peripheral innervation was disrupted from P7 on in cutaneous, visceral and deep peripheral tissues, supporting that the population of Drg11-dependent primary afferent neurons is not tissue specific. However, visceral tissues were much more affected, which agrees with c-fibers making up the bulk of visceral sensory innervation (Cervero, 1985).

Taken together, the data showed that Drg11 is required for the maintenance, immediately after birth, of a significant fraction of normally differentiated small-diameter, putative nociceptive peptidergic and non-peptidergic primary afferent neurons mainly belonging in the C-unmyelinated class and innervating all peripheral tissues. These neurons are likely to follow programmed cell death in the absence of Drg11, as indicated by an increase at P7 of the immunoreactivity for an active form of caspase-3, an ubiquitous caspase that is a main effector of the apoptotic cascade (for review, see Yuan and Yankner, 2000).

DRG11 COMMANDS THE DIFFERENTIATION OF NOCICEPTIVE SPINAL NEURONS

The correct perception of noxious events relies on the activation of distinct sensory neurons specifically organized in different laminae in the spinal cord dorsal horn. These neurons differentiate during development in a spatial-temporal order due to the expression of combinatorial sets of homeodomain transcription factors. *Drg11*$^{-/-}$ mice exhibit defects in the superficial dorsal horn similar to those observed in *Tlx3/1* or *Lmx1b* knockout mice (Cheng et al., 2004, 2005; Ding et al., 2004), suggesting that the three transcription factors belong to a genetic cascade involved in building up the spinal cord superficial nociceptive circuit (Gross et al., 2002; Muller et al., 2002; Qian et al., 2002).

The present thesis shows that various subpopulations of superficial dorsal horn neurons can be defined by the differential combination of Drg11, Tlx3 and Lmx1b (***Publication IV***). During embryonic development, Drg11-immunoreactivity was detected both in early-born (dI3 and dI5) and late-born glutamatergic (dILB) Tlx3/Lmx1b-positive neurons. All newly formed early-born Drg11-positive neurons expressed the glutamatergic fate determinant gene, Tlx3 (Cheng et al., 2004; our own results). Postnatally, four subpopulations were identified. Although the majority (85%) expressed both Tlx3 and Lmx1b with (58%) or without (27%) Drg11, a small fraction (15%) did not express Tlx3. This is in line with the observation by Xu and collaborators (2008) of some lamina III neurons Tlx3-dependent during development that did not express Tlx3 after birth. Half (7%) of the Tlx3-negative neurons only expressed Drg11. Drg11-positive neurons spanned the entire superficial dorsal horn from laminae I to III, although prevailing in lamina II (65%). The subpopulation that expressed Lmx1b but not Tlx3 (7%) was however located in lamina III.

In the *Drg11*$^{-/-}$ spinal cord, the majority of Tlx3- and Lmx1b-positive neurons was absent from E18.5 on. This observation agrees with the detection of abnormal cell death at E17.5 (***Publication II***) and further reveals the glutamatergic nature of *Drg11-/-* dependent neurons. However, it is worth noting that there is a significant fraction of glutamatergic neurons that are not Drg11-dependent, which is preserved in the *Drg11*$^{-/-}$ mice in amounts similar to those present in wild-type mice. The same is true for the GABAergic, Pax2-positive population. Both findings support the assumption that Drg11-dependent neurons degenerate instead of following another differentiation pathway.

The non Drg11-dependent superficial dorsal horn neurons were confined to a narrow strand, which could be delineated in spinal slices silver impregnated by the Golgi-Rio Hortega method (***Publication IV***). In these preparations, a clear reduction in the number of small size, spiny super-

ficial dorsal horn neurons was observed, which was more marked than would be expected from the sum of glutamatergic non Drg11-dependent neurons and GABAergic, Pax2-dependent neurons. The deep dorsal horn did not exhibit any changes nor presented small spiny neurons, which is against the possibility that, due to reorganization of the dorsal horn, superficial dorsal horn neurons were relocated in the deep dorsal horn. That discrepancy in numbers is more probably due to the fact that spinal neurons stained by the Golgi method represent a very small fraction of the entire neuronal population.

There was also a marked reduction in the numbers of Fos-positive neurons in the superficial dorsal horn following noxious stimulation (***Publication IV***). In the deep dorsal horn, c-fos induction was identical to that observed in wild-type mice. This finding, while in line with the normal development of the deep dorsal horn, raises important questions as to the role of superficial glutamatergic local circuit neurons in the nociceptive activation of deep dorsal horn neurons. Although the spared - mainly Aδ - primary afferent neurons can account for the activation of the deep dorsal horn, the spinal cord pain modulatory circuitry of *Drg11*$^{-/-}$ mice must be deprived of an important excitatory component. This should result on an imbalance favouring inhibition of nociceptive transmission, and consequently on the decrease of noxious-evoked c-fos activation in the deep dorsal horn. In this respect, it is worth noting that about 85% of lamina II local circuit neurons are glutamatergic (Santos et al., 2007, 2009), against 20% GABAergic neurons, as revealed by the present work. It is possible that the spinal and supraspinal pain control systems have adapted along development to re-establish the lost balance.

On the other hand, in spite of normal nociceptive activation in the deep dorsal horn, nociceptive behaviour is seriously affected (***Publication II***), which poses interesting questions regarding the relative role of the lamina I and deep dorsal horn nociceptive ascending systems in pain processing (for review, see Lima, 2008).

THE RELATIVE CONCENTRATION OF DRG11 AND ITS SPLICE VARIANT ALONG DEVELOPMENT MAY CONTRIBUTE TO ITS DIFFERENTIAL ROLE IN THE DRG AND SPINAL CORD

Gene expression can be differentially regulated by splice variants, providing a mechanism for precise control of diverse morphogenetic events (Grabowski and Black, 2001; Fagnani et al., 2007; Li et al., 2007; Irimia et al., 2009). Here, we have characterized a Drg11 alternative splice variant (also known as Prrxl1-b), which lacks the OAR domain (***Publication V***). To date, the function of the OAR domain is not properly known but it is believed to have a molecular function directly related to the transcriptional activity of the paired-like homeodomain proteins (Simeone et al. 1994; Galliot et al., 1999; Meijlink et al., 1999; Norris and Kerne, 2001).

The Drg11 splice isoform presented the same regional distribution pattern along the entire neuroaxis as Drg11, but differed as to its relative quantitative expression profile in the DRG and spinal cord at distinct developmental ages. The amount of Drg11 was higher than that of its splice variant at both sites. However, in the DRG the two isoforms exhibited relatively high levels of expression, with the same temporal profile and a similar ratio from prenatal to postnatal ages. In the spinal cord, the expression of the Drg11 splice variant was practically nil from E18.5 on, with a striking high ratio between the two isoforms, particularly at P0. These observations suggest that the two isoforms are differentially regulated in the DRG and spinal cord, and raise the hypothesis that tissue-specific control of the amount of Drg11 relative to its splice variant is a key factor for the regulation of the molecular mechanisms that govern the development of the nociceptive circuit, and may contribute to the differential role of this transcription factor in the two regions (see below). Considering the marked down-regulation of the Drg11 splice variant in the spinal cord between E18.5 and P7, a time interval that encompasses the embryonic age at which synaptic connectivity is

occurring (Fitzgerald, 2005), it is possible that it acts as a repressor of synaptic organization at the superficial dorsal horn.

THE ROLE OF DRG11 IN THE DEVELOPMENT OF THE PRIMARY AFFERENT - SPINAL NOCICEPTIVE CIRCUIT

The work developed in this thesis departed from the hypothesis, raised by the study of Saito and collaborators (1995), that Drg11 should be important for the normal differentiation and synaptic connection of primary afferent and spinal cord nociceptive neurons. The loss-of-function studies performed allowed us to conclude that indeed Drg11 is crucial for the formation of a major component of superficial dorsal horn glutamatergic neurons (***Publication IV***), but apparently irrelevant for the normal development of primary afferent nociceptive neurons (***Publication III***). Peripheral targeting of primary afferent neurons also developed normally in the absence of Drg11 (***Publication III***), whereas spinal targeting appeared to be disrupted, since central projections had difficulty in entering the spinal grey and finding their proper termination area (***Publication II***). Normally, primary afferent fibers arrive at the dorsal root entry zone by E10.5 and begin to invade the spinal gray matter at E12.5 (Ozaki and Snider, 1997). In the absence of Drg11, primary afferent arrival at the entry zone and penetration into the spinal gray was delayed by 3 to 4 days, and their distribution in the superficial dorsal horn somehow disrupted, conforming the abnormal dorsal horn morphology installed at this age (***Publication II***). Later, shortly after birth, these primary afferent neurons, which until then were phenotypically normal, underwent apoptosis and their numbers were markedly reduced, with parallel impairment of peripheral innervation (***Publication III***).

These data strongly support the hypothesis that primary afferent neurons died due to the fact that they did not find their target neurons in the dorsal horn. It is known that neuronal survival requires trophic support, which depends on the establishment of correct connections with the targets (Snider and Silos-Santiago, 1996; Kirstein and Fariñas, 2002; Markus et al., 2002). In the rat, primary afferent neurons establish functional contacts with spinal neurons and peripheral tissues next to birth (Fitzgerald and Fukton, 1992; Hall et al., 1997; Jackman and Fiztgerald, 2000; Fitzgerald, 2005). At neonatal age, peripheral innervation was apparently normal in the absence of Drg11, which leaves the observed loss of 2/3 of glutamatergic superficial dorsal horn neurons as the putative cause for target deprivation-induced primary afferent neuronal death. It is worth noting that, besides the sealed 1/3 glutamatergic neurons, superficial GABAergic neurons and deep dorsal horn nociceptive neurons were also preserved and may have accounted for the conservation of 2/3 of thin primary afferent peptidergic and non-peptidergic neurons in the knockout mice.

However, although following normal differentiation (***Publication III***), and in spite of normal neurogenesis at the superficial dorsal horn until E14.5 (***Publication IV***), in the absence of Drg11, primary afferent neurons had trouble in finding their way to the spinal cord at ages as early as E10.5, which points to a role for this transcription factor in very early stages of guiding primary axons to their spinal targets. It is known that, during development, DRG neurons extend their axons toward the dorsolateral part of the spinal cord, enter the spinal cord at the dorsal root entry zone and then grow longitudinally inside the spinal cord to form the dorsal funiculus without penetrating the dorsal mantle layer (for review see, Masuda and Shiga, 2005). Only after a 'waiting period' of a few days do these axons project into the dorsal mantle layer in a ventral to dorsal order. Proprioceptive afferents are the first to send collaterals ventrally, followed by large-caliber sensory afferent and at last fine calibre nociceptive and thermoreceptive afferents (Ozaki and Snider, 1997). Inhibitory cues transiently expressed, such as Sema3a (Messersmith et al., 1995; Shepherd et al., 1997; Fu et al., 2000; Masuda et al., 2003) and Netrin-1 (Watanabe et al., 2006; Masuda et al., 2008; Masuda et al., 2009) are apparently required for the correct patterning of sensory afferents during this waiting period (Ozaki and Snider, 1997). Furthermore, there is increasing evidence that trans-

cription factors and cell surface molecules, such as Runx3, Er81, Pea3 and F11, are involved in the correct projection of proprioceptive DRG axons (Arber et al., 2000; Perrin et al., 2001; Inoue et al., 2002; Livet et al., 2002; Chen et al., 2006), supporting evidence that several molecules orchestrate in order to elaborate the waiting period for sensory afferents. Appropriate neuronal migration is a prerequisite for the normal projection of primary afferents to the developing spinal cord. Ding and collaborators (2005) have shown that migration of early-born neurons is essential for the central projection of primary afferents, since they repel nociceptive and chemoattract proprioceptive afferents probably through the activity of Sema3a.

To our best knowledge, Drg11 is the sole transcription factor that was shown to be present in migrating superficial nociceptive neurons, to account for their normal differentiation and to be involved in the correct projection of nociceptive afferents into the spinal cord (present thesis). It is however very probable that other transcription factors contribute to this process. A careful analysis of the genetic program under the control of Drg11 is being carried out in our laboratory in order to identify new players that, together with Drg11, govern the establishment of the first relay of the ascending nociceptive system.

In summary, the data collected in this thesis suggest that Drg11 plays a double role in the formation of the DRG-spinal nociceptive circuit. It is involved in the differentiation of a major subpopulation of excitatory nociceptive superficial dorsal horn neurons in one hand, while apparently commanding the guiding and synaptic connectivity of their primary afferent pathway, on the other. Future studies using conditional Drg11 mutations should be conducted to test this hypothesis.

REFERENCES

Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell 101: 485-498.
Cervero F (1985). Visceral nociception: peripheral and central aspects of visceral nociceptive systems. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 308: 325-337.
Cervero F, Connell LA (1984). Fine afferent fibers from viscera do not terminate in the substancia gelatinosa of the thoracic spinal cord. Brain Res. 294: 370-374.
Chen AI, de Nooij JC, Jessell TM (2006). Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. Neuron 49: 395-408.
Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, Arata S, Shirasawa S, Bouchard M, Luo P, Chen C-L, Busslinger M, Goulding M, Onimaru H, Ma Q (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci 7: 510-517.
Cheng L, Samad OA, Xu Y, Mizuguchi R, Luo P, Shirasawa S, Goulding M, Ma Q (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat Neurosci 8: 1510-1515.
Ding YQ, Yin J, Xu HM, Jacquim MF, Chen ZF (2003). Formation of whisker related principal sensory nucleus-based lemniscal pathway requires a paired homeodomain transcription factor, Drg11. J. Neurosci. 23: 7246–7254.
Ding YQ, Yin J, Kania A, Zhao ZQ, Johnson RL, Chen ZF (2004). Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. Development 131: 3693-3703.
Ding YQ, Kim JY, Xu YS, Rao Y, Chen ZF (2005). Ventral migration of early-born neurons requires Dcc and is essencial for the projections of primary afferents in the spinal cord. Development 132: 2047-2056.
Ernfors P, Lee KF, Kucera J, Jaenisch R (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell 77: 503-512.
Fagnani M, Barash Y, Ip JY, Misquitta C, Pan Q, Saltzman AL, Shai O, Lee L, Rozenhek A, Mohammad N, Willaime-Morawek S, Babak T, Zhang W, Hughes TR, van der Kooy D, Frey BJ, Blencowe BJ (2007). Functional coordination of alternative splicing in the mammalian central nervous system. Genome Biol. 8: R108.
Fariñas I, Jones KR, Backus C, Wang XY, Reichardt LF (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369: 658-661.
Fitzgerald M, Fulton BP (1992). The physiological properties of developing sensory neurons. In: Scott, S.A., (Ed.), Sensory Neurons: diversity, development and plasticity. Oxford University Press, NY, pp. 287-306.
Fitzgerald M (2005). The development of nociceptive circuits. Nat. Rev. Neurosci. 6: 507-520.
Fu SY, Sharma K, Luo Y, Raper JA, Frank E (2000). SEMA3A regulates developing sensory projections in the chicken spinal cord. J. Neurobiol. 45: 227-236.
Fundin BT, Arvidsson J, Aldskogius H, Johansson O, Rice SN, Rice FL (1997). Comprehensive immunofluorescence and lectin binding analysis of intervibrissal fur innervation in the mystacial pad of the rat. J. Comp. Neurol. 385: 185-206.
Galliot B, de Vargas C, Miller D (1999). Evolution of homeobox genes: Q50 Paired-like genes founded the Paired class. Dev. Genes Evol. 209: 186-197.
Grabowski PJ, Black DL (2001). Alternative RNA splicing in the nervous system. Prog. Neurobiol. 65: 289-308.
Gross MK, Dottori M, Goulding M (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34: 535-549.
Hall AK, Ai X, Hickman GE, MacPhedran SE, Nduaguba CO, Robertson CP (1997). The generation of neuronal heterogeneity in a rat sensory ganglia. J. Neurosci. 17: 2775-2784.
Inoue K, Ozaki S, Shiga T, Ito K, Masuda T, Okado N, Iseda T, Kawaguchi S, Ogawa M, Bae SC, Yamashita N, Itohara S, Kudo N, Ito Y (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat. Neurosci. 5: 946-954.
Irimia M, Rukov JL, Roy SW, Vinther J, Garcia-Fernandez J (2009). Quantitative regulation of alternative splicing in evolution and development. Bioessays 31: 40-50.
Jackman A, Fitzgerald M (2000). Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. J. Comp. Neurol. 418: 281-298.
Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R,Bryant S, Zhang L, Snider WD, Barbacid M (1994). Disruption of the neurotrophin-3 receptor gene trkC eliminates Ia muscle afferents and results in abnormal movements. Nature 368: 249-251.
Kirstein M, Fariñas I (2002). Sensing life: regulation of sensory neuron survival by neurotrophins. Cell. Mol. Life Sci. 59: 1787-1802.
Li Q, Lee JA, Black DL (2007). Neuronal regulation of alternative pre-mRNA splicing. Nat. Rev. Neurosci. 8: 819-831.
Light AR, Perl ER (1979a). Reexamination of the dorsal root projection to the spinal dorsal horn including observation on the differential termination of coarse and fine fibers. J. Comp. Neurol. 186: 117-132.
Light AR, Perl ER (1979b). Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J. Comp. Neurol. 186:133-150.
Lima D (2008). Ascending pathways: Anatomy and Physiology. In The Senses: A comprehensive reference. Basbaum, A. et al., editors. pp.477-526, Elsevier, London
Livet J, Sigrist M, Stroebel S, De Paola V, Price SR, Henderson CR, Jessell TM, Arber S (2002). ETS gene Pea3 controls the central position and terminal arborisation of specific motor neuron pools. Neuron 35: 877-892.
Markus A, Patel TD, Snider WD (2002). Neurotrophic factors and axonal growth. Curr. Opin. Neurobiol. 12: 523-531.
Masuda T, Shiga T (2005). Chemorepulsion and cell adhesion molecules in patterning initial trajectories of sensory axons. Neurosci. Res. 51:337-347.
Masuda T, Tsuji H, Taniguchi M, Yagi T, Tessier-Lavigne M, Fujisawa H, Okado N, Shiga T (2003). Differential non-target-derived repulsive signals play a critical role in shaping initial axonal growth of dorsal root ganglion neurons. Dev. Biol. 254: 289-302.
Masuda T, Watanabe K, Sakuma C, Ikenaka K, Ono K, Yaginuma H (2008). Netrin-1 acts as a repulsive guidance cue for sensory axonal projections towards the spinal cord. J. Neurosci. 28: 10380-10385.
Masuda T, Yaginuma H, Sakuma C, Ono K (2009). Netrin-1 signaling for sensory axons. Cell Adhesion and Migration 3:171-173.
McCarthy PW, Lawson SN (1990). Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene related peptide-like immunoreactivity. Neuroscience, 34: 623-632.
Meijlink F, Beverdam A, Brouwer A, Oosterveen TC, Berge DT (1999). Vertebrate aristaless-related genes. Int. J. Dev. Biol. 43: 651-663.

Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14: 949-959.
Müller T, Brohmann H, Pierani A, Heppenstall PA, Lewin GR, Jessell TM, Birchmeier C (2002). The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34: 551-562.
Norris RA, Kern MJ (2001). Identification of domains mediating transcription, repression, and inhibition in the paired-related homeobox protein, Prx2 (S8). DNA Cell Biol. 20: 89-99.
Ozaki S, Snider WD (1997). Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J. Comp. Neurol. 380: 215-229.
Perrin FE, Rathjen FG, Stoeckli ET (2001). Distinct subpopulations of sensory afferents require F11 or axonin-1 for growth to their target layers within the spinal cord of the chick. Neuron 30: 707-723.
Qian Y, Shirasawa S, Chen C-L, Cheng L, Ma Q (2002). Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1. Genes Dev 16: 1220-1233.
Saito T, Greenwood A, Sun Q, Anderson DJ (1995). Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol. Cell. Neurosci. 6: 280-292.
Santos SF, Rebelo S, Derkach VA, Safronov BV (2007). Excitatory interneurons dominate sensory processing in the spinal substantia gelatinosa of rat. J Physiol. 581:241-254.
Santos SF, Luz LL, Szucs P, Lima D, Derkach VA, Safronov BV (2009). Transmission efficacy and plasticity in glutamatergic synapses formed by excitatory interneurons of the substantia gelatinosa in the rat spinal cord. PLos One 4: e8047.
Shepherd IT, Luo Y, Lefcort F, Reichardt LF, Raper JA (1997). A sensory axon repellent secreted from ventral spinal cord explants is neutralized by antibodies raised against collapsing-1. Development 124: 1377-1385.
Snider WD, Silos-Santiago I (1996). Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. Philos. Trans. R. Soc. Lond. B 351: 395-403.
Simeone A, D'Apice MR, Nigro V, Casanova J, Graziani F, Acampora D, Avantaggiato V (1994). Orthopedia, a novel homeobox-containing gene expressed in the developing CNS of both mouse and Drosophila. Neuron 13: 83-101.
Watanabe K, Tamamaki N, Furuta T, Ackerman S, Ikenaka K, Ono K (2006). Dorsally derived netrin 1 provides an inhibitory cue and elaborates the 'waiting period' for primary sensory axons in the developing spinal cord. Development 133: 1379-1387.
Willis WD, Coggeshall RE (1991). Sensory mechanisms of the spinal cord. Plenum Press, NY and London.
Willis, WD, Westlund, KN, Carlton, SM (2004). Pain system. In: The rat nervous system, Third edition, Edited by Paxinos. Chapter 27:853-890.
Xu Y, Lopes C, Qian Y, Liu Y, Cheng L, Goulding M, Turner EE, Lima D, Ma Q (2008). Tlx1 and Tlx3 coordinate specification of dorsal horn pain-modulatory peptidergic neurons. J Neurosci. 28: 4037-46.
Yuan J, Yankner BA (2000). Apoptosis in the nervous system. Nature 407: 802-809.
Zwick M, Davis BM, Woodbury J, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002). Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J. Neurosci. 22: 4057-4065.

IV

SUMMARY AND CONCLUSIONS

In this work, we investigated the putative involvement of Drg11 in the development of the nociceptive system, in particular at the DRG-spinal level, as suggested by the study by Saito and Collaborators (1995) showing that this transcription factor is expressed in the DRG and spinal superficial dorsal horn.

Through the manufacture of a polyclonal antibody against DGR11, we were able to demonstrate that Drg11 is expressed throughout embryonic development, from as early as the embryonic age 10.5 (E10.5) until early postnatal age, in first and second order sensory structures along the entire neuroaxis (***Publication I***). At the spinal level, DRG11 immunostainig was observed in small diameter DRG neurons and the superficial dorsal horn, while at the supraspinal level, it was observed in several cranial sensory ganglia and the respective relay nuclei in the brainstem. This distribution pointed out a putative role for DRG11 in the formation of the first link of the ascending sensory pathway, not only at the spinal level but also along the brain.

In order to ascertain whether DRG11 specifically commands the development of the nociceptive system, we underwent the phenotypic characterization of a Drg11 knockout mice (*Drg11*$^{-/-}$) (***Publication II***). *Drg11*$^{-/-}$ mice exhibited reduced reflex responses to mechanical, thermal and chemical painful stimuli, together with anatomical and neurochemical abnormalities in the superficial dorsal horn and misdistribution of their primary afferents, which entered the spinal gray with a marked delay.

We then addressed the involvement of Drg11 in the development of primary afferent nociceptive neurons (***Publication III***) and observed that, in mice deprived of Drg11, primary afferent neurons follow normal differentiation and project normally to their peripheral targets until neonatal age. Shortly after birth, however, about 1/3 both of peptidergic and non-peptidergic, putative nociceptive neurons followed apoptosis, which paralleled marked impairment of peripheral innervation of the skin, viscera and deep tissues. These data indicate that Drg11 is not required for the normal differentiation of small diameter, putative nociceptive primary afferents, but essential for the survival of a significant fraction of those neurons immediately after birth.

The analysis of the differentiation of spinal cord dorsal horn neurons in wild type and *Drg11*$^{-/-}$ mice followed (***Publication IV***). Drg11 was shown to be required for the differentiation, after E 14.5, of a subset, amounting to 73%, of nociceptive glutamatergic superficial dorsal horn neurons, which could be subdivided in 3 different categories expressing either (i) Drg11, Tlx3 and Lmx1b, (ii) Drg11 and Lmx1b, or (iii) Drg11 alone. Moreover, Golgi studies confirmed the absence, in *Drg11*$^{-/-}$- mice, of a large amount of small, spiny neurons in the spinal superficial cord dorsal horn, while *c-fos* induction studies revealed defective noxious-evoked activation at the superficial but not the deep dorsal horn. Besides demonstrating that Drg11 is required for the proper development of a major fraction of glutamatergic superficial dorsal horn neurons, these data indicate that the lack of this excitatory, mostly local circuit neuronal population does not result in decreased nociceptive activation at the deep dorsal horn, which, together with the depressed nociceptive behavior observed in these animals (***Publication II***), underlies the importance of the spinal superficial nociceptive relay in pain processing. In addition, taking into account the normal embryonic development of primary afferents in the absence of Drg11, reported in ***publication IV***, these data support the hypothesis that postnatal death of primary afferent neurons in this condition is accounted for by the lack of a neuronal target in the spinal gray.

Lastly, we investigated the contribution of a Drg11 splice variant to the observed differential role of Drg11 in the DRG and spinal cord (***Publication V***). We verified that both Drg11 and its isoform are present in both regions along development, although a marked decrease in the relative concentration of the DRG11 isoform takes place after E18.5 in the spinal cord, but not in the DRG. This finding suggested a role for the Drg11 isoform as a repressor of the establishment of synaptic connections between primary afferents and superficial dorsal horn neurons, which is known to take place between E18.5 and birth.

Altogether the studies that compose this thesis unravel a role for Drg11 in the development of the first arm of the ascending nociceptive pathway, and reveal that Drg11 may both command the differentiation of a large fraction of glutamatergic nociceptive superficial dorsal horn neurons and the establishment of its afferent connections from the DRG.

V

RESUMO E CONCLUSÕES

Neste trabalho, investigámos o envolvimento do gene Drg11 no desenvolvimento do sistema nociceptivo, nomeadamente ao nível do gânglio raquidiano e medula espinhal, tal como sugerido pelo estudo de Saito e colaboradores (1995) que mostrava que este factor de transcrição é expresso no gânglio raquidiano e no corno dorsal superficial da medula espinhal.

Através da produção de um anticorpo policlonal contra DGR11, demonstrámos que o Drg11 é expresso durante o desenvolvimento embrionário, logo a partir da idade embrionária 10,5 (E10.5) até a idade pós-natal precoce, em estruturas de primeira e segunda ordem sensorial ao longo da neuroeixo (***Publicação I***). Ao nível da medula espinhal, o Drg11 foi observado por imunohistoquímica em neurónios pequenos do gânglio raquidiano e nas lâminas superficiais da medula espinhal, enquanto que ao nível supra-espinhal, observou-se no gânglio sensorial do trigémio e em vários núcleos de projecção no tronco cerebral. Essa distribuição sugeriu um possível papel do Drg11 na formação da primeira ligação da via ascendente sensorial, não só a nível espinhal, como também ao longo do encéfalo.

A fim de se verificar se Drg11 está envolvido especificamente no desenvolvimento do sistema nociceptivo, realizámos a caracterização fenotípica de ratinhos knockout Drg11 (*Drg11*$^{-/-}$) (***Publicação II***). Ratinhos *Drg11*$^{-/-}$ apresentaram uma redução das respostas a estímulos dolorosos de natureza mecânica, térmica e química, juntamente com alterações anatómicas e neuroquímicas ao nível do corno dorsal superficial, para além uma má distribuição dos seus aferentes primários, que entravam na substância cinzenta da medula espinhal com um atraso significativo.

Em seguida, observámos o papel do Drg11 no desenvolvimento de neurónios nociceptivos aferentes primários (***Publicação III***) e observámos que, em ratinhos *Drg11*$^{-/-}$, os neurónios aferentes primários diferenciavam-se normalmente e projectavam normalmente para os seus alvos periféricos até a idade neonatal. No entanto, após o nascimento, cerca de 1/3 dos neurónios nociceptivos peptidérgicos e não peptidérgicos sofriam apoptose que era seguida de deficiente inervação periférica da pele, vísceras e dos tecidos profundos. Estes dados indicam que o Drg11 não é necessário para a diferenciação normal de neurónios nociceptivos aferentes primários, mas é essencial para a sobrevivência de uma parcela significativa desses neurónios imediatamente após o nascimento.

Seguiu-se a análise diferencial de neurónios das lâminas superficiais do corno dorsal da medula espinhal em ratinhos *Drg11*$^{-/-}$ e wildtype (***Publicação IV***). O Drg11 mostrou-se necessário para a diferenciação de uma subpopulação, depois de E14,5, que correspondia a 73% dos neurónios glutamatérgicos presentes no corno dorsal, e que podem ser subdivididos em três categorias diferentes que expressam tanto (i) Drg11, Tlx3 e Lmx1b, (ii) Drg11 e Lmx1b, (iii) apenas Drg11. Além disso, realizámos estudos com impregnação pelo método de Golgi Rio-Hortega que confirmaram a ausência, em ratinhos *Drg11*$^{-/-}$, de uma grande quantidade de pequenos neurónios espinhosos no corno dorsal superficial da medula espinhal, enquanto que estudos de indução do protooncogene *c-fos* revelou uma diminuída activação no corno dorsal superficial mas não no corno dorsal profundo, após estimulação nóxica. Além de demonstrar que Drg11 é necessário para o bom desenvolvimento de uma grande fracção de neurónios glutamatérgicos do corno dorsal, estes dados indicam que a falta desta população excitatória não resulta em diminuição da activação nociceptiva no corno dorsal profundo, que juntamente com a resposta comportamental diminuída após estimulação dolorosa observada nestes animais (***publicação II***), reforça a importância das lâminas superficiais no processamento nociceptivo. Além disso, tendo em conta o desenvolvimento embrionário normal dos aferentes primários, na ausência de Drg11, mencionado na ***publicação IV***, esses dados apoiam a hipótese de que a morte pós-natal dos neurónios aferentes primários nesta condição é explicada pela falta de um alvo neuronal espinhal.

Finalmente, investigámos a contribuição de uma isoforma do Drg11 no papel diferencial do Drg11 observado no gânglio raquidiano e da medula espinhal (***Publicação V***). Verificámos que tanto o Drg11 como a sua isoforma estão presentes em ambas as regiões ao longo do desenvolvimento, apesar de uma diminuição acentuada na concentração relativa da isoforma Drg11 que ocorre após E18.5 na medula espinhal, mas não no gânglio raquidiano. Este achado sugere um papel para a isoforma Drg11 como um repressor do estabelecimento de conexões sinápticas entre os aferentes primários e os neurónios superficiais do corno dorsal, que se sabe ocorrer entre E18.5 e o nascimento.

No seu conjunto, os estudos que compõem esta tese, permitem revelar um papel para Drg11 no desenvolvimento do primeiro componente da via nociceptiva ascendente, e revelam que o Drg11 é necessário para a diferenciação de uma grande fracção de neurónios glutamatérgicos nociceptivos presentes nas lâminas superficiais do corno dorsal e no estabelecimento de sinapses com o gânglio.