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ABSTRACT

Several neurotransmitter systems have been related to developmental processes during the past decade. In this review, we discuss the evidence that the nicotinic acetylcholine receptors could have an additional function during development that may be unrelated to their role in cholinergic neurotransmission in the vertebrate brain. Both temporal expression data and *in vitro* and *in vivo* studies with nicotinic agonists and antagonists have provided direct support for a role of nicotinic receptors in neural developmental processes such as neurite outgrowth and differentiation. A similar picture has emerged for other neurotransmitter and receptor systems as well, which generates a new view of neural processes during both development and mature life.

Key words: acetylcholine, development, neurotransmitters, nicotine, nicotinic receptors.

INTRODUCTION

Considerable progress has been obtained in understanding the processes that control the development of the nervous system. Notable advances have been mainly generated by molecular biological approaches, which revealed several key aspects of neural development, including the determination of cell identities, target reaching, and synaptogenesis (Francis and Landis 1999, Hatten 1999). Many genes and molecules have been related to specific developmental processes. Among the recently studied developmentally-related molecules are those that control the specification and differentiation of nervous system subdivisions, such as the *homeobox*, *engrailed*, *wnt* and *pax* gene products (Joyner 1996,

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Maconochie et al. 1996), those that regulate neurite outgrowth and axon targeting, such as gangliosides, ephrins, semaphorins, and SNARE proteins (Flanagan and Vanderhaegen 1998, Mueller 1999, O´Leary and Wilkinson 1999, Hepp and Langley 2001, Mendez-Otero and Santiago 2001), and those that regulate neuron survival and plasticity, such as the neurotrophins (Reichardt and Fariñas 1997, Shatz 1997, Huang and Reichardt 2001).

During the past decade another group of molecules has been clearly related to developmental regulation, namely the neurotransmitters and their receptors. This previously unsuspected role of the neurotransmitter systems has been revealed by experiments using *in vitro* preparations of several neuronal tissues and showed that neurotransmitters such as serotonin, dopamine, glutamate and acetylcholine were able to promote or block neurite outgrowth, de-

pending on the neuronal group and the neurotransmitter involved (for review see Lipton and Kater 1989). For example, an inhibition of growth cone motility in cultured retinal neurons was observed after dopamine treatment and this effect was thought to depend on the D1-type dopamine receptor (Lankford et al. 1988). At about the same time, the N-methyl-D-aspartate-type glutamate receptors have been unequivocally linked to the precise patterning of connections during nervous system development (Constantine-Paton et al. 1990, Shatz 1990, Simon et al. 1992). These plasticity events, namely neurite sprouting, establishment of neuronal connections, and exclusion of connections, profoundly affect the general organization of the developing and the mature nervous system. There is now consensus that those neurotransmitter systems, together with the gamma-amino-butyric acid (Billinton et al. 2001) and the nitrinergic (Contestabile 2000) systems, are all involved in some aspect of neural development, in addition to their well-known role in neurotransmission. In this review we focus on the possible involvement of nicotinic acetylcholine receptors (nAChRs) in developmental processes such as neurite outgrowth and differentiation.

THE NICOTINIC RECEPTORS

The nAChRs are members of the neurotransmitter-gated superfamily of ion channels and are all formed by combinations of five subunits organized around a central ion channel. The structure of nAChRs have actually provided the model for understanding of the organization of neurotransmitter-gated ion channel receptors (Lindstrom 1998). In the nervous system, twelve distinct nAChR subunits have been cloned and identified as α ($\alpha2$-$\alpha10$) and β ($\beta2$-$\beta4$) subunits, which may constitute heteromeric or homomeric receptors. The distinct combinations of nAChR subunits produce receptors with different physiological properties and both α and β subunits seem to contribute to the functional diversity of the nAChRs.

At least two branches of nAChRs may be defined based mainly on their affinity for the snake venom α-bungarotoxin (α-Bgt), which are the α-Bgt-sensitive and α-Bgt-insensitive nAChRs. The major brain subtype with high affinity for nicotine seems to be the $\alpha4\beta2$ nAChR (Flores et al. 1992), which is α-Bgt-insensitive. The major ganglionic subtype is $\alpha3\beta4$ and the major subtype with high affinity for α-Bgt found in brain and ganglia is composed of homomeric arrangements of $\alpha7$ (Del Toro et al. 1994). The α-Bgt-sensitive nAChRs, especially the $\alpha7$-containing subtype, have been the target of intense investigation as they show high calcium permeability (Vijayaraghavan et al. 1992, Séguéla et al. 1993, Rathouz et al. 1996). Calcium entering the neuron through $\alpha7$ channels could then act as a second messenger in several cellular processes, such as the regulation of neurite outgrowth and therefore of developmental and plasticity processes (Quik 1995).

The expression of nAChR subunit genes in *Xenopus* oocytes provided a great deal of information about the organization and function of those receptors, but it was necessary to verify if functional combinations obtained *in vitro* resembled the subtypes expressed in the nervous system. Ligands, antibodies and RNA probes have all been employed for the localization and quantification of the neuronal nAChRs. For example, the localization of the nAChRs have been investigated in vertebrate brains with ligands (Clarke et al. 1985, Sorenson and Chiappinelli 1992), immunohistochemistry to detect the nAChR proteins (Sargent et al. 1989, Hamassaki-Britto et al. 1991, Britto et al. 1992a,b, Torrão et al. 1997) and *in situ* hybridization to detect the corresponding mRNAs (Goldman et al. 1986, 1987, Boulter et al. 1987, Duvoisin et al. 1989, Wada et al. 1989, 1990, Morris et al. 1990, Dineley-Miller and Patrick 1992, Séguéla et al. 1993, Lohmann et al. 2000).

All these studies converged to reveal that the nAChRs are widely distributed throughout the mature nervous system. The next obvious step was to analyze the expression of different nAChR subunits during development of the nervous system.

nAChR EXPRESSION DURING DEVELOPMENT

Several studies have shown an early expression of nAChRs and of the cholinergic system enzymes, acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) in the central and peripheral nervous system of vertebrates (see Role and Berg 1996, Torrão et al. 2000). These studies employed either *in situ* hybridization, immunohistochemistry, immunoblotting, and binding assays, and all these techniques have revealed that the subunits which form the nAChRs are expressed very precociously. Moreover, the observation of different temporal patterns of nAChR expression was another strong indication of the presumptive participation of nAChRs in developmental processes (Daubas et al. 1990, Matter et al. 1990, Zoli et al. 1995, Conroy and Berg 1998, Hellstrom-Lindahl et al. 1998, Kaneko et al. 1998, Torrão et al. 2000). The study by Conroy and Berg (1998) shows, for example, an increased expression of $\alpha5$, $\alpha8$, and $\beta2$ nAChR subunits between embryonic day 8 [E8] and E17/18 in the chick brain. Similar patterns are observed in the rat brain (Zoli et al. 1995). Transient expression is another pattern observed for several nAChR subunits. This pattern was detected in our laboratory for several subunits in the chick cerebellum (Fig. 1). The $\alpha2$, $\alpha5$, $\alpha7$, $\alpha8$, and $\beta2$ nAChR subunits all begin to be expressed relatively late (at E12), show a peak of expression at E16, and are expressed at very low levels or not expressed at all in the postnatal chick (Kaneko et al. 1998, Torrão et al. 2000). Very early expression of nAChRs has also been documented in the chick retina, where some types of nAChR appear at least two days before choline acetyltransferase is detected and about four days before the onset of synaptogenesis (Hamassaki-Britto et al. 1994).

EFFECTS OF NICOTINIC AGONISTS AND ANTAGONISTS UPON NEURAL DEVELOPMENT IN VITRO

Functional *in vitro* studies have been also conducted to assess the role of nAChRs during development. Primary cultures of neuron precursors or cultures of stable cell lineages have been analyzed to observe the relationship between nAChR expression and processes such as proliferation, cell survival and neurite outgrowth. The addition of the nicotinic agonist nicotine in PC12 cells and ciliary ganglion cultures produced a decreased rate of growth or even a retraction of neurites in both cases, which is in agreement with the popular idea of a growth inhibitory effect of nicotine in the developing central nervous system. This effect was reverted by application of α-Bgt, which indicates that the effect is mediated through α-Bgt-binding nAChRs (Chan and Quik 1993, Pugh and Berg 1994). Indeed, a developmental role of the nAChRs has been mainly suggested for the α-Bgt-binding nAChR subtypes. These studies have suggested that neurite outgrowth regulation could be a result of an activation of the α-Bgt-binding nAChR subtypes, which characteristically show a high calcium permeability. In addition to increasing the levels of intracellular calcium by fluxing the ion directly through the receptor channel, the activation of α-Bgt-binding nAChR subtypes may activate voltage-gated calcium channels *in vivo* and then increment the calcium influx. Interestingly, a recent study has shown an opposite effect of nicotine. In this study, the addition of the nicotinic agonists to cultured olfactory bulb neurons produced significant increases in neuritic length, an effect that was abolished by treatment with α-Bgt, indicating the involvement of $\alpha7$-containing nAChRs in this process. This hypothesis was confirmed by binding assays with ^{125}I-α-Bgt (Coronas et al. 2000). These conflicting results could be related to different calcium concentrations in those cell culture preparations. It has been suggested that an optimal level of intracellular calcium is necessary for neuritic extension and outgrowth (Kater and Mills 1991) and it seems that these levels differ between neurons with spontaneous neurite outgrowth (PC12 cells and ciliary ganglion cells) and neurons with little neuritic extension under basal conditions (olfactory bulb neurons).

In the context of the above considerations, it is

nAChR subunits	Stages					
	E10	E12	E14	E16	E18	Adult
α2	absent	slight	moderate	intense	moderate	slight
α3	absent	intense	moderate	absent	absent	absent
α5	absent	slight	moderate	intense	moderate	slight
α7	absent	slight	moderate	intense	moderate	slight
α8	absent	absent	slight	moderate	slight	absent
β2	absent	slight	slight	intense	slight	absent

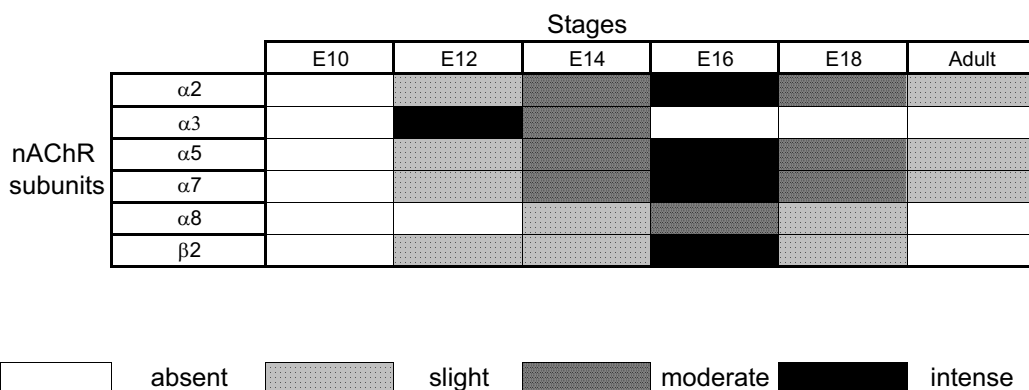absent   slight   moderate   intense

Fig. 1 – Temporal evolution of immunoreactivity for nAChR subunits in Purkinje cells of the developing chick cerebellum. Note that the expression of most subunits tested peak around E16. The α3 subunit shows a peak of expression four days earlier.

also relevant that the treatment of primary cerebellar neuroblasts of rats with nicotine increased proliferation and cell survival (Opanashuk et al. 2001). Also, primary cell cultures from human fetal brain treated with nicotine showed an up-regulation of nAChR binding sites and increased expression of their transcripts (Hellstrom-Lindahl et al. 2001).

EFFECTS OF NICOTINE UPON *IN VIVO* NEURAL DEVELOPMENT

The effects of nicotine treatment upon the developing brain has also been assessed with *in vivo* studies. Nicotine treatment during the second postnatal week generated a disruption of the synaptic development of the rat auditory cortex (Aramakis et al. 2000). Also, rats treated with nicotine during the second postnatal week showed a persistent increase in the number of nAChR binding sites in the adult stage (Miao et al. 1998). These two studies indicate that the second postnatal week is critical in the developing rat brain and that nicotine treatment during this period could induce permanent effects on nAChRs. In addition, prenatal or embryonic nicotine exposure has been conducted to investigate its effects upon fetal development. These studies have investigated the morphological, functional, and behavioural changes that may be produced by early exposure to nicotine (e.g. Pennington et al. 1994).

Some studies have also addressed the question of nicotine-mediated effects upon the nAChR themselves and upon cholinoceptive neurons, which are presumptively the targets of nicotine in the brain. Prenatal nicotine exposure transiently increased the levels of α4, α7, and β2 mRNA in hippocampus and cortex of postnatal rat brain (Shacka and Robinson 1998). On the other hand, the chronic infusion of nicotine in chick eggs did not change the density of (-)-[^{3}H]nicotine-binding sites in the developing chick brain (Roll et al. 1993). We have found in our laboratory that the infusion of nicotine in chick eggs seems to produce a reduction of immunoreactivity for the α2, α5, α8, and β2 nAChR subunits in perikarya and neuropil of specific regions of the chick embryonic brain, with an apparent disruption of the neuritic structure of neurons containing those subunits. In contrast, the infusion of α-Bgt during specific stages of embryogenesis seemed to generate an increase of immunoreactivity for the α8 nAChR subunit. The dendritic arbors of the cholinoceptive neurons appeared to be more profusely ramified or at least much more visible when the cells were stained for the presence of that nAChR subunit (Fig. 2). This result suggests that early during development the nAChRs are responsive to nicotinic agonists and antagonists, and the development of nicotinic receptor-containing neurons may be regu-

lated by blockade and activation of those nAChRs. The increased neuritic immunoreactivity for the $\alpha 8$ nAChR subunit and the enriched dendritic arbor observed after α-Bgt infusion seem to agree with previous in vitro studies that have already implicated the α-Bgt-sensitive nAChRs in morphogenetic processes (Chan and Quik 1993, Pugh and Berg 1994, Role and Berg 1996). The apparent facilitation of neurite development *in ovo* could be the result of an optimal calcium concentration obtained after the presumptive blockade of $\alpha 8$-containing receptors by α-Bgt.

CONCLUDING REMARKS

The above data suggest that nAChRs, especially those of the α-Bgt-type branch, may control the development of neural structures in which they are expressed. A possible mechanism for such a control could be the termination of axonal growth by presynaptic nAChRs that flux calcium. Acetylcholine released from growth cones might be responsible for the activation of those nAChRs. In addition, these receptors may influence the establishment of the dendritic morphology, which is suggested by both *in vivo* and *in vitro* experiments. Finally, it is noteworthy that these same mechanisms may operate during plastic remodeling in the adult brain (Dani et al. 2001). Taken together, the data reviewed here indicate that the cholinergic and other chemically-specific brain systems participate in neural development and plasticity, in addition to mediating neuro-transmission in the adult brain.
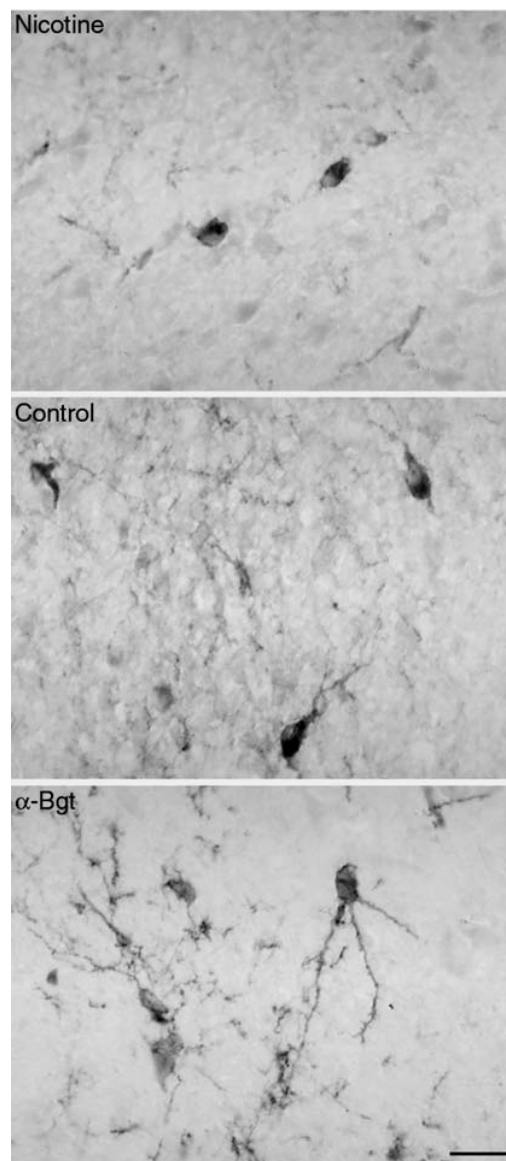
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Fig. 2 – Effects of *in ovo* injections of nicotine and α-Bgt upon $\alpha 8$-like immunoreactivity in neurons of the chick lateral mesencephalic reticular formation. Dendritic staining is much more evident after α-Bgt. Nicotine, on the other hand, appears to reduce neuropil staining for $\alpha 8$. Control eggs were injected with either saline or distilled water. All embryos were treated from E15 through E17 with daily injections, and were sacrificed on E18. Scale bar: 25μm.

RESUMO

Na última década vários sistemas de neurotransmissores têm sido relacionados ao controle do desenvolvimento do sistema nervoso. Nesta revisão, nós discutimos as evidências de que os receptores nicotínicos da acetilcolina podem apresentar uma função incomum durante o desenvolvimento, que não deve estar relacionada com seu papel na neurotransmissão colinérgica no sistema nervoso de vertebrados. Dados sobre expressão temporal e estudos *in vitro* e *in vivo* com agonistas e antagonistas nicotínicos têm proporcionado apoio direto para a hipótese de uma função dos receptores nicotínicos no desenvolvimento neural, incluindo processos como crescimento neurítico e diferenciação. Um quadro similar tem sido obtido para outros sistemas de neurotransmissores e receptores, o que tem gerado uma nova visão dos processos neurais durante o desenvolvimento e a vida adulta.

Palavras-chave: acetilcolina, desenvolvimento, neurotransmissores, nicotina, receptores nicotínicos.

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