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Abstract

We have investigated the role of N-methyl-D-aspartate receptors (NMDARs) and γ-aminobutyric acid receptors type A ($GABA_ARs$) at an early DIV 2-4 stage of P19 neuronal differentiation. With the use of RT-PCR we have verified the expression of various NMDAR and $GABA_AR$ in the process of P19 neuronal differentiation and we established the functionality of these receptors via fluo-3 Ca^{2+} imaging. In immature DIV 2 P19 neurons both NMDA and GABA excited the neuronal bodies, but only polyamine-site sensitive NMDAR stimulation led to enhanced Ca^{2+} signaling in the growth cones. Inhibition of NR1/NR2B NMDARs by 1 μM ifenprodil severely impaired P19 neurite extension and fasciculation, and this negative effect was fully reversible by 50 μM polyamine (spermine or spermidine). In contrast, $GABA_AR$ antagonism by a high dose of 200 μM bicuculline had no observable effect on P19 neuronal differentiation and fasciculation. Except for the differential NMDAR profile of Ca^{2+} signaling within the immature DIV 2 P19 neurons, we have also shown that inhibition of NR1/NR2B NMDARs strongly decreased mRNA level of NCAM-180, which is well-known regulator of neuronal growth cone protrusion and neurite extension. Our data thus suggest a critical role of NR1/NR2B NMDARs during the process of neuritogenesis and fasciculation of P19 neurons via differential control of local growth cone Ca^{2+} surges and/or NCAM-180 signaling.

Introduction

Both N-methyl-D-aspartate receptors (NMDARs) and γ-aminobutyric acid receptors type A ($GABA_ARs$) are expressed early in neuronal development and regulate neural progenitor proliferation, neuronal migration and neuronal differentiation. More interestingly, the corresponding neurotransmitters glutamate and GABA are paracrinely released via SNARE-independent mechanism at an early neuronal developmental stage even before synapse formation and both neurotransmitters lead to depolarization of maturing neurons and increase of intracellular Ca^{2+} levels, while in mature neurons GABA leads to hyperpolarization and suppresses Ca^{2+} influx. Maturation of the neuronal phenotype includes changes both in neuronal excitability and morphology as well as establishment of appropriate connectivity within the new formed electric circuits, and electrical activity is emerging as a key modulator of neuronal development, a fact that underlies the increasing interest in elucidating the relative importance of NMDARs and $GABA_ARs$ in neurogenesis and the precise timing of their actions. Since both NMDARs and $GABA_ARs$ are functionally expressed in differentiated mouse P19 neurons, and in P19 neuronal cultures there are both excitatory (glutamatergic) and inhibitory (GABAergic) synapses, we have decided to address the NMDAR vs $GABA_AR$ role in neuronal differentiation using P19 neuronal model.

NR2B NMDARs control P19 neurite fasciculation

The main finding in this study is the involvement of NR1/NR2B NMDARs in neurite extension and fasciculation at an early DIV 2-4 stage of P19 neuronal differentiation. This was shown by pharmacological blockade of NR1/NR2B NMDARs with 1 μM ifenprodil for DIV 2-4, which lead to severe thinning of neurite fascicles, which are normally present in DIV 4 P19 neuronal networks. Moreover the observed ifenprodil effect was reversible by 50 μM polyamine application (spermine or spermidine), which restored the neurite fascicle parameters back to the control levels. Similar to ifenprodil effect was observed also by application of 20 μM of the non-selective NMDAR ion channel blocker MK-801.

NR2B NMDARs are located in growth cone filopodia

In order to understand better the effects of NMDAR in the process of neuritogenesis we decided to perform functional study of the NMDAR mediated Ca^{2+} fluxes in neuronal bodies, neurites and growth cones. Fluo-3 Ca^{2+} imaging experiments established that NMDARs are located in growth cone filopodia

of DIV 2 P19 neurons. NMDAR mediated Ca^{2+} fluxes in developing growth cones were sensitive to ifenprodil and polyamines arguing for presence of NR2B subunit in the molecular composition of these nonsynaptic NMDAR channels. Though the localized Ca^{2+} entry points in filopodia were found to be insensitive to diltiazem pretreatment, the calcium collection in the central part of the growth cone was sensitive to VGCC inhibition (schematic representation of our findings is presented in Figure 1). The finding of functional NR2B NMDARs in the growth cones suggests that NMDARs could mediate at least partially the attractive effects of glutamate in axon guidance.

NR2B NMDARs control NCAM splicing

Considering that NMDAR dependent Ca^{2+} fluxes in growth cones alone might not explain the effect of NMDARs on neurite fasciculation, we tried to identify effector molecules acting downstream of NMDAR Ca^{2+} influx. With the use of quantitative RT-PCR we have uncovered polyamine site dependent NMDAR mediated splicing control of NCAM mRNA, in which NR2B NMDAR activation promotes inclusion of exon E18 producing the NCAM-180 isoform. Additonally mRNA levels for GAP-43, MAP-2 and N-cadherin were not significantly changed, which argues against possible non-specific downregulation of NCAM-180 as a secondary effect of decreasing the number of neurite fascicles in ifenprodil and MK-801 treated P19 cultures. Since NCAM molecules were previously implicated in the process of neurite fasciculation, we concluded that NCAM-180 is at least one of the effector molecules downstream of NMDAR Ca^{2+} signaling, which regulates neurite differentiation.

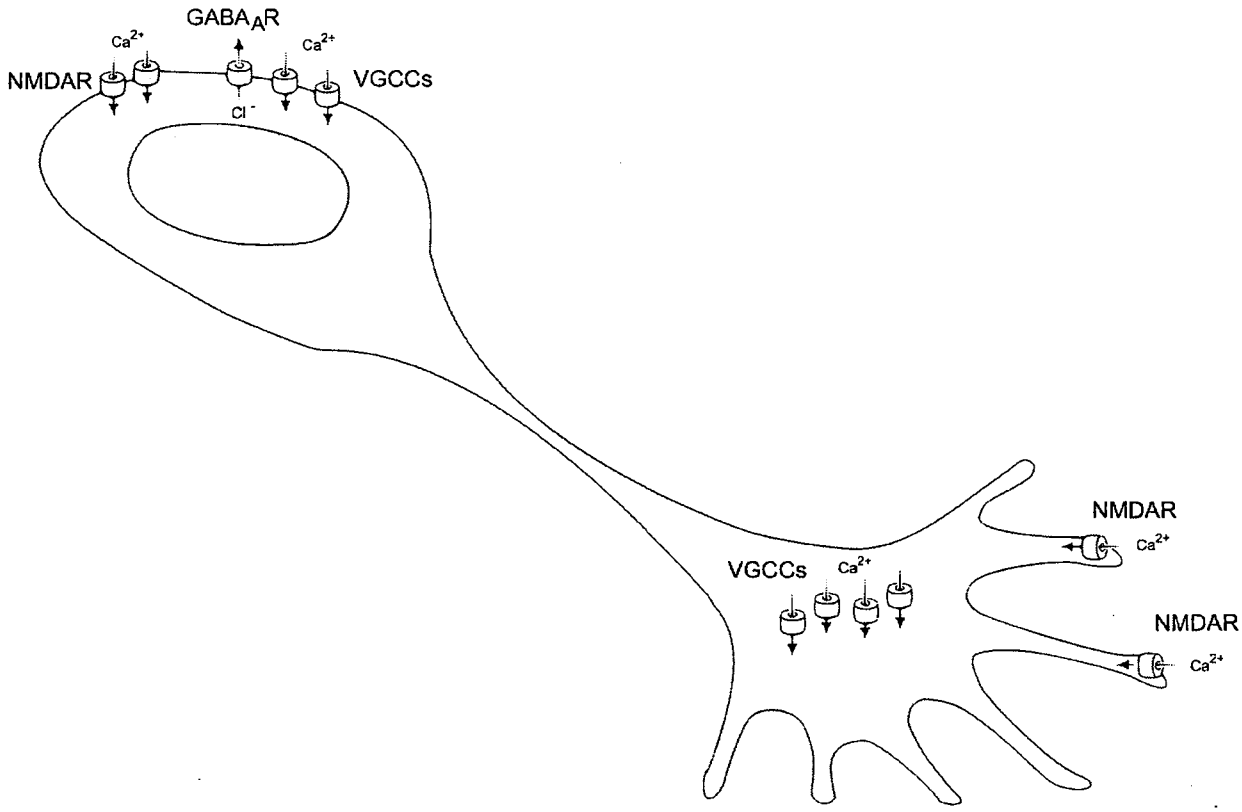

Figure 1. Proposed model of NMDAR and GABA$_A$R Ca^{2+} signaling in maturing P19 neurons based on our Fluo-3 Ca^{2+} imaging data.

GABA$_A$R block does not impair P19 neuronal differentiation

We have shown early expression and functional activity of GABA$_A$Rs in immature DIV 2 P19 neurons. Moreover we have found that GABA$_A$R activation by 400 μM GABA leads to Ca^{2+} influx in neuronal bodies via coupled VGCCs, which sense the changes in the neuronal membrane potential, however in contrast to NMDAR stimulation, GABA application was not able to trigger significant change in growth cone Ca^{2+} levels (see Figure 1). VGCC involvement in GABA induced Ca^{2+} influx was verified by usage of 100 μM bicuculline or 100 μM diltiazem, both of which were able to complete abolish the response to 400 μM GABA. GABA$_A$R blockade by 200 μM bicuculline for DIV 2-4 impaired neither P19 neuronal differentiation nor P19 neurite fasciculation providing evidence that glutamate but not GABA is critical for the proper development of the P19 neuronal network.

Discussion

With several minor modifications of previously existing protocols we were able to investigate both P19 neuronal differentiation at the single neuron level as well as P19 supraneuronal network organization in the form of star-like neuronal clusters communicating via thick-rope like neurite fascicles containing one or more axonal or dendrite trunks. In our in vitro model we were able to assess the expression profiles of NMDAR and $GABA_AR$ subunits and monitor the functional role of those receptors by pharmacological blockade or stimulation via applied antagonists or agonists. The data from the performed experiments suggests that NMDAR but not $GABA_AR$ function is critical for early stage of P19 neurogenesis. Though we have provided evidence for the critical role of NMDAR in neurite fasciculation in P19 networks in vitro, still the question how these data apply to real neurons in real life remains open. There are several approaches available that have been tried in order to elucidate the NMDAR role in neuritogenesis such as in vivo imaging of GFP-expressing optic tectal neurons from an intact anesthetized Xenopus tadpoles, studying of organotypic explans or comparing the brain development of genetically modified and wild type mice. Therefore it is desirable that the observed role of NR2B NMDAR in neurite outgrowth and fasciculation is verified in other in vitro model systems or in vivo.

学位論文審査結果の要旨

本研究では、神経細胞発生初期過程における神経性アミノ酸の機能的役割を解明する目的で、増殖能と分化能を有するマウス胚性がん細胞株 P19 細胞を用いて、脳内の代表的なイオノトロピック型受容体である NMDA 受容体および $GABA_A$ 受容体活性化の影響について解析した。

P19 細胞をレチノイン酸存在下に浮遊条件下で培養すると、4 日目までは培養日数に比例して大きな細胞塊の形成が確認された。この細胞塊を分散後に接着条件下で再度培養すると、その後 2 日目までには神経細胞マーカータンパク質である MAP2 陽性細胞が多数出現したが、アストログリア細胞マーカータンパク質である GFAP 陽性細胞の出現は観察されなかった。分散培養直後の細胞には機能的 NMDA チャネル構築に必須の NR1 サブユニットと、ヘテロダイマーチャネル形成に必要な NR2A および NR2B サブユニットの mRNA 発現は全て検出されたが、NR2C および NR2D サブユニット発現は培養条件にかかわらず P19 細胞株には認められなかった。一方、$GABA_A$ 受容体については分散後の細胞にα4、α5、β3 およびγ2 など、機能的チャネル構築に必要なサブユニットの mRNA 発現が確認された。分散培養 2 日目の細胞を用いて、細胞内遊離 Ca^{2+} 濃度を測定したところ、NMDA 曝露に伴って遊離 Ca^{2+} 濃度の著明な上昇が見られたが、この上昇は NR2B サブユニット阻害作用を有する ifenprodil によって消失した。このときに spermine と spermidine をさらに添加すると ifenprodil の効果は拮抗されたのに対して、GABA 曝露に伴う Ca^{2+} 濃度上昇は $GABA_A$ 受容体アンタゴニストの bicuculline によって消失した。分散培養開始直後から ifenprodil を添加すると、NMDA 受容体アンタゴニストである MK-801 の場合と同様に、その後の神経突起伸張や神経線維束形成が強く阻害されたが、この ifenprodil による阻害は spermine や spermidine の添加によって回復した。さらに、ifenprodil 曝露細胞では細胞接着因子の一つである NCAM180 の mRNA 発現の有意な低下が招来されたが、この低下は spermine や spermidine の添加によって回復した。したがって、NMDA 受容体は少なくとも一部は NCAM180 発現調節を通じて、神経突起伸張や神経線維束形成などのシナプス形成に関与する可能性が示唆される。

以上の研究成績は、神経細胞発生過程におけるシナプス形成メカニズムに NMDA 受容体が関与する可能性を提唱するだけでなく、今後の神経細胞機能制御を指向する創薬戦略展開への貢献が期待される点で、薬理学的および分子生物学的に高く評価されるので、審査委員会は本論文が博士（薬学）に値すると判断する。